

9 April 1981

Per Ardua ad Astra (et Terram)

One swallow does not make a summer, but for people yearning to see the winter end, the sight of even a single swallow is bound to seem a cheerful omen. This is the spirit in which the managers of the United States National Aeronautics and Space Administration (NASA) will be glued to their television screens for the next few days, waiting to see whether the new reusable spacecraft, the shuttle, will succeed. With luck, the first version of the shuttle (called Columbia) will be launched on Friday this week, roughly two years later than first planned. Soon afterwards, it should be clear whether the spacecraft has found its way into an orbit about the Earth. Just over two days later, it may have found its way back again, to an airstrip in California. That is the plan. NASA officials will not be the only people biting their nails through this tense period. Several crucial scientific enterprises, the Large Space Telescope in particular, hang on the success of the shuttle. So do several important technical projects, the further development of the international telecommunications network for example. And while it is by no means clear what the Pentagon has in mind for this new means of putting things and people in orbit, the chances are that a success for Columbia this weekend will spark off a round of vigorous jockeying for priority in the launching timetable among the potential users. So much is easily predictable.

It is therefore churlish but also necessary, even at this late stage, to raise the question of what happens if Columbia fails. Ordinarily, if the shuttle were a more conventional spacecraft, the consequences of failure would be negligible. The managers at NASA would bite their lips, utter whatever stoical comments the circumstances of failure required — and set to on the readying of the next spacecraft from the production line. If Columbia fails, they will no doubt follow the same tack but with less conviction. With the budget for the next fiscal year still enmeshed in Congress, it is unlikely that they could escape the thorough investigation of the whole shuttle programme that they have managed to avoid in the past two years. And even if the first flight of Columbia is a success, it is now clear that the development of the shuttle has been too much of a scramble for comfort.

Of the problems that have beset (and delayed) development,

the most serious is that concerned with the device for protecting the spacecraft from heat on reentry into the atmosphere. Recurring troubles with the ceramic tiles intended to accomplish this would have been comic if they were not also almost tragic. It has been forgotten that the tiles are a makeshift substitute for the wonder alloys foreseen when the shuttle was originally conceived of a decade ago, and which have not materialized. The other glaring deficiency in the development programme is the lack of flight experience. A single launching from the back of a Boeing 747 can hardly command confidence. By contrast, recurring problems with the rocket motors, which may nevertheless turn out to be disastrous, are largely a monument to the American passion for optimizing everything — never use a rocket motor off the shelf if there is a chance that a purpose-built motor might be slightly more efficient seems to be the motto. If Columbia fails this weekend, these questions are certain to be reopened.

Others than NASA officials should therefore keep their fingers crossed. The whole device may have been an imperfectly considered technological leap in the dark, but success promises a quantum jump in the utilization of orbits about the Earth. The planned reduction of cost (by roughly a factor of ten for unit mass placed in an orbit) is only a part of the promise. The possibility that the shuttle may be a means of maintaining and repairing satellites already in orbit is potentially more important. Whatever the outcome of this weekend's experiment, there is therefore the strongest possible case for NASA to spend a substantial part of its hard-won budget on a more satisfactory solution than the present of the heat-shielding problem. That cannot be a simple task. Heat-shields based on thermal insulators whose surfaces ablate at high temperatures, widely used to protect the warheads of long-range missiles, are plainly unsuitable for spacecraft intended to be used repeatedly. Solid insulators intended to get rid of heat by radiation are even more susceptible than others to destruction by thermal shock. But mechanical cooling systems based on circulating fluids are probably impractical — their mass would be too great. NASA has already let one contract, seeking an alternative solution. It should quickly cast its net wider. For the shuttle, for all its defects, it is the best American spacecraft in sight.

Who, not Congress, should police fraud?

How often do scientists cheat? How seriously, in the process, is the scientific literature corrupted? And what, if anything, should be done to put a stop to the occasional transgressions that come to light? The United States Congress (see page 435) should not be surprised that it failed to answer these questions in its brief inquiry last week into a few of the cases of wrongdoing that have come to light in the past few months. On the other hand, there is no reason why the research establishment should resent the inquiry or even fear that the outcome will be a novel, intrusive and probably unworkable system of regulation imposed from Washington. There is no reason to suppose that Representative Albert Gore's subcommittee seeks to be a kind of arbiter of ethics in the research laboratory or that it plans to recommend some central system of regulation. The most sinister of the plausible motives for last week's inquiry is that the subcommittee, which plans next to take up more general (and important) issues such as the propriety of arrangements between academic scientists and commercial companies in the field of genetic engineering, the adequacy of agreed procedures for experiments with human subjects and the

like, is seeking to demonstrate at the outset that not all scientists are, like Caesar's wife, above reproach.

The committee's inquiry would have been more productive if it had more clearly thought through the problem of malfeasance in research before dragging its impressive collection of witnesses to Washington last week. It should not have confined its inquiry to biomedical research, and it should have distinguished more clearly between the different kinds of misdemeanours that have from time to time become open scandals. The committee (and those who gave evidence last week) should also have had the wit to recognize that any formal central system for the prevention of misdemeanours is likely also further to inhibit freedom of access to the scientific literature. Journals and their referees are already sufficiently aware of the dangers of having wool pulled over their eyes. More restraint would diminish the chance that heterodox ideas would find their way into the light of day and further tip the scales against innovations.

Plagiarism, the most flagrant of misdemeanours, is paradoxically not as serious a cause for concern as some pretend.



The activities of Dr E.A.K. Alsabti which came to light last year (*Nature* 285, 429 and 286, 437; 1981) were a flamboyant demonstration of how a person can make a mark in the literature with the help of a photocopying machine and a little ingenuity in rewriting the titles of other people's articles. Of necessity, however, success (if that is the word) requires that the plagiarist should be satisfied with publication in relatively obscure journals and that he should pass off as his own only uncontroversial or even dull observations by other people. Journals are not well-placed to spot this kind of transgression — even though some of Alsabti's published papers should have set off alarms in the editorial offices of the journals in which they appeared. But even the most ingenious plagiarist cannot expect a long career. However obscure the journal in which he publishes, and however uninteresting the topic, systematic retrieval systems are now good enough to ensure that those working in the field, including the authors whose work has been plundered, will recognize what has happened within a year or so. In more active fields of research, detection is likely to be even faster. So much is clear from the sensation last year in which one of Professor Philip Felig's collaborators at Yale University was accused of having stolen some text from a paper he had seen as a referee: not unnaturally, the plagiarizing paper was sent to the original authors for review and the whistle was quickly blown. Out-and-out plagiarism, then, is likely to be a nuisance only for librarians and bibliographers. It is exceedingly improbable that working scientists will be misled. And the penalties for the transgressors are severe — they are as a matter of routine expelled from the scientific community.

The falsification of data is necessarily a more troublesome misdemeanour. If a person chooses to send for publication a set of data which is the product of imagination and not experiment, there is very little that journals and their referees can do to identify the deception. Nor would it help if would-be authors were required to send with their manuscripts copies of their laboratory notebooks (even though journals often require that authors should submit evidence to support puzzling conclusions, for example when there are doubts about the statistical analysis of raw data which do not form an integral part of a paper intended for publication). In the ordinary course of business, journals and their referees are not as competent as a reasonably competent investigator to make an assessment of what is involved. The laboratory bench has something in common with the confessional. It must be supposed that a person is telling the truth. If he is not, the penalties are severe, as Dr John Long, dismissed in January 1980 from the Massachusetts General Hospital (see *Nature* 289, 227; 1981) for falsifying published data, has found to his cost.

Unfortunately, cases of falsification rarely emerge as clearly as this. The scientific literature is replete with records of observations which cannot be accommodated within the accepted framework of understanding but which are much more often the result of faulty techniques and mistaken interpretation than of deliberate falsification. And, no doubt, hidden in this mass of data are observations representing genuine challenges to accepted theories. It would be catastrophic if journals habitually declined to give house-room to data that could not be accommodated within the existing body of received wisdom. Although, on such occasions, journals have a duty to enquire closely into the methods by which data have been obtained, in the last resort it is essential that uncomfortable data should be published, not suppressed. If some small part of these data should be fabricated — and it is more likely that false data will be used in the support of orthodoxy, which is just as dangerous — that cannot be the end of the world.

In the nature of things, it is exceptional that a single set of data can overthrow an accepted theory or sustain one that should be discarded. Even dramatic events such as the observation in 1921 that the images of distant stars were deflected at the limb of the Sun as predicted in 1917 by Einstein's theory of gravitation did not still (and have not stilled) arguments about the validity of that monumental work. Similarly, Dr Joseph Weber's entirely well-intentioned claim (in 1968) that gravitational waves had been

detected by equipment based at the University of Maryland and the Argonne National Laboratory, although published in respectable journals (*Physics Review Letters* for example), carried little conviction even though it stimulated several attempts to repeat the observations of a phenomenon that should be observable, although not as strongly as Weber claimed. For what it is worth, the more recent observations by Steele and Gorczynski (see *Nature* 289, 678; 1981) of the inheritance of acquired immunological characteristics in mice deserve a place in the scientific literature even if, in the long run, their interpretation turns out to be different from that now given to them. At least where data bear on general understanding, witchhunting for fabricated data is likely to do more harm than good. Time will tell what data make sense, while the scales are already too powerfully weighted against the heterodox.

So is there nothing for the US Congress to be alarmed about? That, unfortunately, is not the case. Last week's inquiry produced some disturbing evidence of deliberate falsification in the trial and assessment of new drugs. No doubt from the best of motives (a concern that individual patients should have the advantage of the newest drugs), physicians are tempted to include in clinical trials people not eligible under the protocols laid down. Such deceptions will usually lead to the underestimation of the value of a new drug or other treatment, and thus risk the deprivation to future patients of valuable procedures. The safeguard, however, lies in the hands of those who supervise clinical trials — given the understandable temptation, why not demand authenticated medical histories of the patients included in them? Falsification of data bearing on the safety of new drugs, frequently (in the United States) produced by independent contracting laboratories, is potentially a more serious and intractable problem, but here again the remedy is accessible, and administrative. In such circumstances, bodies such as the Food and Drug Administration should (and frequently do) demand access to original laboratory records. Moreover, the agency has sufficiently competent technical staff to carry out a proper assessment of such data. In any case, the laboratories concerned, knowing that their reputations are at risk, have a vested interest in accurate reporting. It should not be beyond the wit of regulatory agencies to ensure that they are not wilfully misled.

So should Representative Albert Gore's committee conclude that there is nothing to be said? That is what Dr Philip Handler was suggesting last week. Unfortunately, that is too simple a view. Although the scientific community is enviably free from wrongdoing, at least as compared with other professional communities (lawyers or politicians, for example), it is too simple to suppose that the peer review system can be relied upon, at least in the long run, to root out all deception. One obvious snag is that acclaimed success, even if based on the over-interpretation of data, tends to breed more success. Grant-making bodies would be less than human if they were able to avoid all such traps. The past few months have also uncovered disturbing evidence that investigators holding substantial research grants are relatively immune from the scientific criticism of their close colleagues, and that they may also be so heavily engaged in the administration of a research programme (including the search for further grants) that they have little time to give to the detailed assessment of the data produced by research programmes in which they are titularly involved. Many of those to whom these descriptions apply will no doubt have mended their ways after learning of the tragic object lessons of the recent past. For the ultimate safeguard of the authenticity of scientific data rests not merely on the peer review system (whose critics necessarily lack first-hand knowledge) but also on full and frank discussion within individual laboratories. The benefits, too often denied, are not merely that a tiny proportion of unauthentic data will struggle with more difficulty into print but that laboratories will in general be made more productive and creative. It would be seemly if the subcommittee in its report urged such steps. It would be ridiculous to go further, and to ask that there should be some formal external process for demonstrating what is already self-evident — that the research profession is as much like Caesar's wife as can be expected.

Universities complain at Pentagon policy

Restrictions on foreign students cause alarm

Washington

Government interference in academic research, ostensibly to control the export of technical data, has stung the presidents of five top universities to protest. The Pentagon is seeking to limit the involvement of foreign research students on certain projects. The universities have also protested, in a letter to Defense Secretary Casper Weinberger and Secretary of State Alexander Haig at attempts to restrict the publication of unclassified research sponsored by the Pentagon.

The university presidents express concern about guidelines issued recently by the department in which defence officials suggest that universities should limit the involvement of foreign students in research related to the development of very high speed integrated circuits (*Nature* 289, 736; 1981). They complain that at least one university has been told by the Department of Commerce not to allow certain foreign scholars to participate in its sponsored research activities — a reference to the Export Administration Regulations restricting the transfer of technical data and equipment to communist nations. More action along these lines is expected.

The signatories to the letter are Donald Kennedy, president of Stanford University; Marvin L. Goldberger, president of the California Institute of Technology; Paul E. Gray, president of the Massachusetts Institute of Technology; Frank H. T. Rhodes, president of Cornell University; and David S. Saxon, president of the University of California. According to the text of the letter, not yet officially released by the universities, the full-scale classification of research, where appropriate, is "far preferable" to the application of "restrictive and virtually unenforceable regulations" to universities. For those university activities which remain unclassified, they add, the government should give up all attempts to apply the restrictions until broader issues — which include a possible conflict with the first amendment to the constitution, guaranteeing freedom of speech — have been resolved.

Two sets of export restrictions lie at the heart of the universities' concern. The first is the International Traffic in Arms Regulations, introduced by Congress in the early 1970s to limit the export to any country of technology (including technical knowhow) of potential military value. The

second is the Export Administration Regulations of the Department of Commerce, aimed at limiting the export of technology of commercial value to certain countries, particularly those in Eastern Europe.

Confusion has arisen in universities and scientific organizations — and in the federal agencies themselves — largely because of the difficulty in drawing a clear line between basic knowledge obtained by scientific research and results which, although still described as "basic", have been developed with a particular long-term military or commercial application in mind.

Last year, for example, the regulations were invoked by the Department of Commerce to prevent scientists from Eastern Europe from attending an open meeting on computer bubble memories organized by the American Vacuum Society, one of more than 40 scientific societies which have since applied for exemptions from the export controls for future scientific meetings on the grounds that, since these are open meetings, they should not be considered to involve the transmission of "secret" data.

The concern expressed by the five

university presidents, however, applies more specifically to the conditions that must be observed by scientists in US universities and research institutions, and their freedom to publish the results of their research or to communicate it to non-American scientists through seminars or even informal discussions.

Officials at Cornell University, for example, said last week that in one recent instance the Department of Commerce had placed conditions on a foreign scientist's visit, restricting his access to research at the university, which it had not been prepared to accept, and the visit had been cancelled. In another instance, a research contract proposed by the Department of Defense specified that, although the research was to be unclassified, the department's permission was required before any results were published — a condition which many universities, such as Stanford, say they would find unacceptable.

The five university presidents point out that although the regulations have existed for several years, only recently have they begun to be applied to "traditional university activities". The new interpretation could result in restrictions which

Gowans washes hands of Rothschild

New York

Recent changes in the organization of medical research in the United Kingdom mean that the customer-contractor basis proposed by Lord Rothschild between the Medical Research Council (MRC) and the Department of Health and Social Security (DHSS), "has now been abandoned", Dr James Gowans, director of MRC, told an audience at the Rockefeller University in New York last week.



Gowans — Rothschild off his back

Describing the British approach to medical research funding, Dr Gowans said that after the customer-contractor relationship was introduced in 1972, with substantial funds being transferred from MRC to DHSS, only a handful of new contracts had been awarded. "It became apparent that the customer-contractor

principle was not applicable to medical research, certainly not on the scale envisaged by Rothschild.

Following further discussions, the Rothschild arrangement had been abandoned and the money previously transferred to DHSS had been transferred back to MRC. "We are now back in the situation that we were in before 1972", Dr Gowans said, adding that "to a scientist it is gratifying", since many members of the scientific community had argued strongly against Rothschild's ideas when they first appeared.

MRC and DHSS "both now realize that fundamental solutions to problems such as cancer and coronary heart disease cannot be conjured up by the use of contracts. It is not possible to use bureaucratic devices to accelerate the rate of scientific progress", he added. As part of the new arrangements, MRC had also agreed to undertake more health services research, since the council had been told that much of the work carried out under that heading was very important but not very well done.

Mistakes had arisen in government science policy through a failure to distinguish between areas of research — such as cancer research — where the basic principles were not known and the science could not be substantially accelerated merely by providing additional funding, and areas where the scientific principles were known and additional support would provide more substantial results.

David Dickson

would conflict with the fundamental precepts that define the role and operation of the American university system, they say. Strict interpretation, coupled with the severe criminal penalties that they contain, would be even more restrictive than direct classification of the research.

In response to the letter, whose addressees also include Mr Malcolm Baldrige, the Secretary of Commerce, the three federal agencies involved have apparently told the universities that they are looking closely at the problems raised, but it is expected to be several weeks before a formal reply is agreed upon.

The potential impact of the export control regulations on the academic community is already being discussed by an *ad hoc* committee set up last month by the National Academy of Science's Committee on Science and Public Policy.

The rules are also being closely studied by the National Science Foundation. Dr Donald Langenburg, the Deputy Director, says the foundation is "deeply concerned" about the potential impact of export controls on research, adding that it is "critical" to the freedom of scientific exchange that these are kept to a minimum consistent with foreign policy and national security objectives.

The growing tension between the academic community and the federal agencies over the interpretation of export restrictions is precisely the type of problem for a presidential science adviser, still absent from the White House. **David Dickson**

DNA research guidelines

Dutch get tough

Brussels

The publication by an *ad hoc* committee of revised safety rules for recombinant DNA research has raised some grumbles in Dutch industrial circles. By and large the new guidelines, the conclusions of a year's study of the risks of gene manipulation, follow closely those set by the National Institutes of Health in the United States. But while other European countries are moving towards a relaxation of the rules, the Dutch have tightened up the restrictions on research with pathogenic microorganisms.

Companies such as Unilever and Gest-Brocades, which are carrying out research into the industrial applications of bioengineering are, however, more frustrated about the administrative checks on research. In the Netherlands, DNA research is first vetted by the *ad hoc* committee, although there is no legal obligation to register the research. Local authorities then have the last word over individual experiments and any new research laboratories.

It is up to the committee, the local authority and university advisory groups to interpret the guidelines as they see fit. Industry complains that it is unnecessarily

restrictive to ask permission to carry out each individual experiment. Unilever has taken some of its projects to Belgium and Gest-Brocades has switched some of its resources to its UK laboratories. In the meantime both expect to wait at least a year for approval on new laboratories. The companies feel that local government authorities are much harder on industry than on universities.

The Netherlands is now thought to have the toughest rules in the world. Public distrust of DNA research caused four members of the *ad hoc* committee to abstain from the final vote on the new guidelines on the grounds that there should be a study of the impact of genetic engineering on society as a whole.

The European Community's Economic and Social Committee is taking the same option. Last July the European Commission brought out its own recommendation on the registration of DNA research, which stressed that over the past three years it had become clear that DNA research is not as dangerous as was originally thought. However, before agreement is reached between the Economic and Social Committee and the European Parliament, a public workshop is to be held in May that will bring together representatives from a cross-section of society to discuss the economic, social and ethical implications of such research.

The Commission's recommendation, if it is adopted, would do little more than establish common definitions and provide the basis for a consensus on registration procedures. However, many researchers increasingly feel that arguments about risk assessments are academic. The bulk of research is carried out in the lowest risk class and very rarely in PIII containment conditions. Thus, the slightly stricter rules proposed by the Dutch are expected to have little impact. **Jasper Becker**

Solar Polar mission

Joint project saved?

The European Space Agency (ESA) is "cautiously optimistic" that something will be salvaged from the International Solar Polar Mission, although in a less ambitious form than originally envisaged. The two-spacecraft mission to study the polar regions of the Sun was threatened last month when the US National Aeronautics and Space Administration (NASA) announced that, to meet budget cuts, it would not be building its spacecraft.

Although it is unlikely that NASA will restore the mission to its original status, angry protests and diplomatic pressure by European embassies in Washington seem to have prompted a compromise. According to ESA, that might involve NASA building a cheaper spacecraft — costing \$40 million rather than the \$100 million originally planned.

Under such a scheme, NASA's space-

Congress redispenses

Washington

A congressional subcommittee has now prepared the ground for restoring the United States half of the Solar Polar Mission. Last week, the space science subcommittee of the House of Representatives Science and Technology Committee voted to make cuts elsewhere in the National Aeronautics and Space Administration's (NASA's) budget for the fiscal year 1982; these cuts made it possible for the subcommittee to restore money for projects such as the Solar Polar Mission (which was given an additional \$15 million) without exceeding the total for the NASA budget of \$6,700 million proposed by Mr Reagan. Since the rearranged budget package will not incur additional costs, House Democrats are hopeful that the pro-space science subcommittee of the Senate Commerce Committee, which votes later this month on NASA's authorization, can also be persuaded to accept reinstatement of the solar spacecraft, perhaps on a smaller scale. **David Dickson**

craft, which would lose a de-spun platform carrying a solar coronagraph for direct imaging of the Sun, would be almost identical with ESA's. Two similar spacecraft would allow measurements of spatially and temporally resolved dynamic phenomena, which would not be possible with only one.

ESA's dismay at NASA's decision to abandon its part of the mission was largely due to the fact that it had already spent most of the money for its spacecraft in European industry. NASA, on the other hand, had delayed building its craft after the planned launch date of the mission was put back last year. If NASA is willing to compromise, ESA will agree to a further launch delay to 1986, even though it would increase the costs. The precise details of the compromise will have to wait until Dr James Beggs, NASA's new director, is installed. **Judy Redfearn**

Biotechnology

Canada takes stock

Washington

After six months of intensive study, a task force set up last summer to look at the development of biotechnology in Canada has recommended that the Canadian government should establish a ten-year National Biotechnology Development Plan, with a budget that would rise to about \$50 million a year. Most of this money would go on research and training; exploitation would remain for the private sector, which the task force recommends should receive significant tax incentives.

The task force was set up by Canada's Minister of State for Science and Technology, Mr John Roberts, and was

headed by Dr Maurice Brossard, director of research at the Institut Armand Frappier in Laval, Quebec (*Nature* **285**, 431; 1980). Although acknowledging that the extra financial investments which it recommends would be a significant financial burden at a time of fiscal constraint, the task force says that the proposed sum "is a very conservative estimate of required federal expenditure".

Three factors are listed which make this expenditure important — the wide range of potential applications of biotechnology, the importance of biotechnology to Canada's future industrial development and "the almost complete lack of existing Canadian biotechnological capability".

On the last of these, the report is disarmingly frank. The second section of the report, "Biotechnology in Canada: An overview", contains two short paragraphs which refer to a "practically non-existent biotechnological industrial base", a rapidly shrinking federal government research capability and a highly fragmented and unfocused university research effort.

To correct this situation, the task force suggests that the graduate and postgraduate training programmes of the Natural Sciences and Engineering Research Council and the Medical Research Council should be increased by \$3 million and \$1.4 million respectively in the next financial year.

Less predictable is the proposal that Canada's immigration policies be revised "to address the immediate shortage of biotechnological skills which are in high demand worldwide".

The government is expected to endorse the proposal that commercial exploitation be achieved through tax incentives rather than direct government investment. The task force has, however, suggested direct government assistance of up to \$6 million to encourage small and medium-sized biotechnological industries. **David Dickson**

European uranium supply

Australia signs up

Brussels

A large slice of Europe's need for uranium over the next 30 years is to be met by a guaranteed supply from Australian ore. Australia and the European Atomic Energy Community (Euratom) last week initialled a draft of a joint nuclear safeguards agreement in Canberra which should enable Europe to buy uranium from Australia. Negotiations began in 1979 and the Canberra meeting was the fifth round. The agreement has been pursued with great determination by Euratom because Australia, with 20 per cent of the western world's uranium reserves, is very important to Europe's supply strategy.

The basic deal, to run for 30 years, is for Australia to guarantee supply of uranium exports in exchange for European

Community exports of nuclear technology and promises that the radioactive material will not be re-exported or used for military purposes.

Similar agreements are also being negotiated with Canada and the United States. The Canadian deal is a renewal of the 1959 agreement which expired last year and has run into difficulties on the issue of "sensitive" nuclear material (uranium enriched above 20 per cent). Negotiations to renew the agreement with the United States have been held up until the Reagan Administration is firmly in the saddle.

Negotiations with Australia went smoothly until the Australian government decided to impose new conditions. These included a commitment by Euratom member states to ensure an efficient monitoring of reprocessing techniques and to finalize an international plutonium storage programme; a promise not to use or store plutonium extracted from Australian uranium in a way that might lead to proliferation; and that the member states should ask for prior permission on a case by case basis to use the material for research or civil purposes.

In effect, then, the Australians are going further than the Nuclear Non-Proliferation Treaty. The Community negotiating team have been trying to establish precise definitions of the circumstances in which prior permission must be obtained. Does, for instance, using nuclear power to drive submarines constitute a peaceful purpose?

Full details of the new agreement, likely to be similar to the Australian deals with China and Japan, will not be divulged until the final text is agreed. **Jasper Becker**

Fraud in research

Congress asks why

Washington

Dr John C. Long, whose resignation from the Massachusetts General Hospital was forced last year after the discovery that he had fabricated research data, went before a congressional committee last week in sackcloth and ashes. The committee chairman, Representative Albert Gore, complimented him for shouldering full responsibility for his acknowledged "wrongdoing and dishonesty". Dr Long said that he liked his new job (as a surgical pathologist at a Detroit Hospital) — and that he had no plans to return to research.

Dr Long's remarkable statement was one of the few misdemeanours that the government oversight committee of the House of Representatives Committee on Science and Technology had been able to assemble for its two-day inquiry into wrongdoing in science. On the second day of the inquiry, officials from the Food and Drug Administration accused the National Cancer Institute of withholding information about the potential side effects of an anti-cancer drug it was testing. But most of the hearing was taken up with several cases of apparent fraud and abuse

that have recently emerged in the scientific literature and the public press.

The data which Dr Long admitted to fabricating were included in a paper published in the *Journal of the National Cancer Institute* (62, 787; 1979) in response to a critical comment by a referee. But Dr Long also admitted that he had concealed from his colleagues that cell lines purportedly derived from patients with Hodgkins's disease had been contaminated with other cells later shown to have been owl monkey cells (Harris *et al. Nature* **289**, 228; 1981).

Nature misrepresented

Washington

In his evidence to the subcommittee, Dr Philip Felig said that two journals, one of them *Nature*, had refused to publish a letter of retraction. Dr Felig said that *Nature's* refusal to publish stemmed from a concern for the personal plight of Dr V. Soman, his collaborator responsible for an act of plagiarism who was afterwards found also to have falsified published data.

After the hearing last week, however, Dr Felig agreed with the editor of *Nature* that his evidence to the subcommittee had been misleading; that *Nature* had agreed to publish the retraction when the audit, then under way, of Dr Soman's laboratory work had been completed; and that at the time (June 1980), he had expressed himself as satisfied with this arrangement.

The chairman of the subcommittee was informed of this conclusion both by the editor and by Dr Felig, and agreed to include a note to this effect in the published record. Dr Felig's intended retraction was in respect of the paper "Regulation of the glucagon receptor by physiological glucagonaemia" by V. Soman and P. Felig, *Nature* **272**, 829-832 (1978).

The force of his confession, however, was blunted by the statement by Dr Ronald Lamont-Havers, his former departmental head at the same hospital, that investigations carried out since Dr Long's departure have shown that other data, previously published, may also have been fabricated.

The subcommittee's objective last week was threefold: to estimate the frequency of wrongdoing in biomedical research, to understand the causes of such behaviour, and to decide if anything should be done.

Most witnesses agreed that falsification and plagiarism in the scientific literature is a rare but persistent phenomenon. Dr Donald Fredrickson, director of the National Institutes of Health (NIH), took some pleasure in telling the committee that Gregor Mendel's research results did not stand up to modern statistical analysis.

But even so, the Food and Drug Administration reported that it has disqualified 42

investigators from work with new drugs since 1966, when procedures were devised to deal with violations of the regulations for the administration of new drugs to patients. There have been 20 disqualifications since 1977, when a more formal "Bioresearch Monitoring Program" was set up. At NIH, Dr Raub said that rules covering the grounds on which a research scientist can be barred from receiving further funding had recently been tightened, and that, although no individual had been disbarred in the history of the institutes, at present five instances were on record where a scientist would have questions raised about his or her past conduct if a further research grant application was received.

The committee seemed to accept that known cases of falsification are the tip of only a small iceberg. The suggestion by Dr Philip Handler, president of the National Academy of Sciences, that in the circumstances the committee should turn its attention to more important problems was not, however, well calculated to win friends. The chairman of the committee put Dr Handler firmly in his place with a declaration that falsification entailed the waste of public money and damaged the public reputation of science; and that if the committee was not satisfied that the research community could police itself, it might have to recommend external regulation.

The committee found its position strengthened by testimony from Dr Alexander Capron, executive director of

the President's Commission for the Study of Ethical Problems in Medicine and Biomedical and Behavioral Research, who recounted the difficulties that the commission was having in obtaining details from the Department of Health and Human Services on the procedures followed in cases of suspected misdemeanour, especially in the treatment of human subjects.

Witnesses differed last week on the motives for falsification. Most of the scientific witnesses held that the pressure of competition for research grants is not an important spur to falsification, but the committee seized on Dr Long's admission that to have shared with his colleagues doubts about the authenticity of his cell cultures would have jeopardized his application for a \$500,000 research grant as evidence to the contrary.

Dr Philip Felig, professor of medicine at Yale University, was closely questioned about the relationship between senior scientists and their colleagues. A case of plagiarism in his own laboratory eventually cost him appointment as dean of the medical school at Columbia University (see *Nature* 285, 429; 1980). He acknowledged that there may be occasions when a collaborator sets out to "please" his senior colleagues, and that the dangers are especially great when the senior person has not himself developed the techniques his collaborators are using. Yale, Felig explained, has now introduced a formal procedure for the independent internal investigation of disputed research results.

Known misdemeanours

Apart from the cases mentioned in the accompanying text, the following is a list of the misdemeanours cited at last week's inquiry by the congressional oversight subcommittee.

University of Kansas (1977). Allegations by two women graduate students that a grant awarded by the National Institutes of Health for anthropological research had been wrongly used for work with human subjects, including the collection of blood samples and genetic counselling. The university established (in 1978) that the work with human subjects had not been cleared with the Institutional Review Board. The investigations (and a civil slander suit by the investigator) continue.

Boston University (1978). Allegations of the falsification of medical records of patients and certain research reports, part of a cooperative study of new cancer treatment financed by the National Institutes of Health (NIH), were substantiated by an internal investigation. The leader of the unit (Dr Marc Straus) resigned on 24 June 1978, followed by his more senior associates, and the Boston University records were expunged from the cumulative records of

the Eastern Collaborative Oncology Group. The investigation continues, but Dr Straus, then at the New York Medical College, Valhalla, was awarded an NIH grant of \$1.3 million over three years in January 1980.

University of California, Los Angeles (1979). Allegations of experimental bone marrow transplants without proper approval from the institutional review board (known as the Human Subjects Protection Committee). The investigations continue.

New York (1978). Inspection by Food and Drug Administration (FDA) revealed "persistent violation" of rules for the administration of novel drugs to human patients by the independent psychiatrist Dr Nathan Kline. Formally debarred by FDA on 13 November 1980.

FDA (various) (1966-81). Total of 42 disqualifications from access to "investigational" drugs, with 18 further cases under investigation. So far, four such investigators have been prosecuted (and one gaoled). Roughly 6 per cent of routine investigations "have led to a more in-depth review". Most violations involve lack of patient consent, inadequate accounting for drugs, violation of protocols and inaccurate (or missing) records.

UK hydraulics research

Deep water ahead

The British government's policy of "privatization" — the sale of public assets — which has already led to the sale of all but a minority stake in the British Aerospace Corporation, now threatens several research laboratories run by the Department of the Environment. The Hydraulics Research Station at Wallingford is the most immediately in danger.

The proposal to make the Hydraulics Research Station self-supporting follows a three-month market survey commissioned by the department and completed last October, in which it was suggested that a potential £1.15 million is available from the private sector. Of this, £0.2 million comes from UK markets, £0.28 million from contracts usually covered by European companies, £0.5 million from offshore projects and the remainder from increasing prices by an arbitrary 10 per cent. The present income is made up of 85 per cent from government and 15 per cent from private contracts.

The market survey suggests that although the UK market is fairly static, the potential for contracts in offshore projects and in the developing countries is considerable. However, a recent survey by the National Maritime Institute foresees a decline in offshore contracts over the next five years. There is also formidable European competition for contracts in developing countries.

The experience of private companies is not encouraging for those at Wallingford. Both the Wimpey Laboratories and the British Hovercraft Corporation have suffered severe financial difficulties. The Water Research Centre, which underwent some sort of privatization following the amalgamation of the Water Pollution Research Laboratory, parts of the Water Resources Board and the Water Research Association, is experiencing similar problems. When it was formed in 1974, the centre was seen by central government as a way of keeping small municipal water undertakings up to the mark. The civil servants involved at first rejected the plan but finally agreed on a package deal. The future of the joint enterprise seemed secure — the centre was financed by its members, with all local and municipal water authorities contributing. In the past year, however, there have been almost 100 redundancies.

Employees at Wallingford now fear that the reputation of the laboratory will be damaged. They also fear that the withdrawal of public funding will inevitably lead to a reduction of background research. However, they see advantages in some sort of halfway "privatization" which would free them from government constraints, particularly on staffing levels, and enable them to take on more contract work. The Government is now

not, however, prepared to consider such a proposal although a meeting has now been arranged for 14 April between the Central trade union and the Department of the Environment. A shadow board is to be set up on behalf of the prospective private company to explore the viability of the enterprise and to prepare employees for privatization — the target being 1 September 1981.

Sara Nash

Europe's directive on drugs

Lords back industry

Proposals from the European Commission to make it easier for entrepreneurs to buy drugs in one country of the European Community and sell them at a profit in another have met with strong opposition. The UK pharmaceutical industry and the House of Lords Select Committee on the European Communities

are at one in condemning the idea as unfair to the companies and unlikely to improve the health services in member states.

The opportunity for this "parallel importing" arises because at the moment price controls for drugs are widely different in the various member states. The European Court of Justice ruled that attempts by big pharmaceutical companies to stop parallel importing were restrictive and in breach of the Treaty of Rome.

Drug manufacturers and importers must obtain marketing authorization from national health services. To make things easier for the parallel importer in the name of free trade, the commission's directive requires the original manufacturer to make available to the importer any necessary documents relating to the specification of the batch of drug being re-sold. This type of freedom of information is anathema to drug companies which may have spent thousands of pounds in obtaining the information in the first place.

In putting the case for the industry, the Association of the British Pharmaceutical Industry (ABPI) argued that moves to bring down the general level of drug prices would not be in the long-term interest of the consumer. And the practice of parallel importing raises problems because importers may change the name and packaging of the drug, with risks that the consumer might not get the correct information about the product.

In the United Kingdom the pricing structure for drugs has kept prices relatively low. In the past few years companies have been allowed to increase prices in line with inflation but for some time "product licences" were given on condition that price increases were kept at about half that rate. As always the industry is keen to stress how expensive it is to develop new drugs and that this should be considered in pricing. So the prospect that the low-price UK market might be opened up to bulk buyers wanting to sell in countries where UK manufacturers are allowed to sell at higher prices is seen as a cause for alarm by both ABPI and the Lords Committee.

The United Kingdom is the most successful exporter in the Community, and last year the British pharmaceutical industry achieved a record £522.9 million surplus of exports over drug imports despite the problems caused by a strong pound. The Lords report sees little reason to jeopardize the well-being of a healthy industry only to give more profits to the middle-men.

The general question of bringing prices into line across the European Community will need more thought than a simple attempt to promote free trade. Both continental and UK manufacturers would favour a uniform system that is subtle enough to take account of both national and international pressures on the companies, and the particular needs and preferences of health services in the member states.

Charles Wenz

Opening up Siberia

Research in steppes

More than 400 scientific research institutes are to be involved in the Soviet Union's new "Siberia" programme which is part of a plan to give this relatively backward area an economic growth rate 30 per cent higher than that of the rest of the country.

Projects will concentrate on the energy sector, according to Academician Valentin Kapyug, chairman of the Siberian section of the Soviet Academy, with "work of world class" on methods of obtaining synthetic liquid fuel from coal and natural gas. The Baikal-Amur Mainline railway has opened up a new zone for potential development and much of the investment will be in that area. Later more industrialization will take place around the Arctic coast, and in zones around new water resources created by the diversion southward of water from the Siberian rivers.

A food development programme includes proposals from 27 biological and research institutes for the development of new varieties of crops and livestock adapted to Siberian conditions,



Siberia, here we come

"hormonal" methods for increasing crop yields, and pest control methods aimed at leaving minimum toxic residues.

Siberia already produces 50 per cent of the oil and 33 per cent of the natural gas extracted in the Soviet Union. The extraction of fossil fuels in Siberia is expected to go on increasing up to the end of the century, so not surprisingly the new "Siberia" programme also contains work on new prospecting and surveying methods, industrial catalysts and forestry management.

Any practical benefits obtained through the new research programme will be concentrated around "technical production complexes" linking several scores of industrial enterprises. One such complex has already been established, based on the giant power stations on the Angara and Yenisei rivers.

Vera Rich

Tangled policy on drugs

Brussels

The European Court of Justice ruled in 1976 that Hoffman-La Roche were impeding free trade in refusing to give product information to parallel importers. The Italian company Centrafarm had been buying Valium in the United Kingdom and repackaging it for resale under the trivial (not registered) name "diazepam".

The latest proposals from the European Commission are another attempt to limit the monopoly power of large drug companies. Opposition to the Commission comes from most members states, and the European Proprietary Association, representing pharmaceutical companies producing drugs for general sale rather than medical prescription, spoke against the proposals at the Lords committee.

Germany, Italy and the Netherlands are considered high-price countries — on several occasions the European Commission has caught out Italy for supporting unrealistically high prices and court actions have brought down prices in Italy by about 20 per cent.

One effort by the Commission to simplify procedures was the Committee for Proprietary Medicinal Products set up five years ago. The committee can be called upon if a producer wants to put a product on the market in at least six member states, or if there are disparities in the judgements of national licensing authorities. But since it was set up the committee has examined only eight cases.

The adverse comments from the House of Lords seem certain to put another nail in the coffin of the Commission's proposal which has already been languishing for a year in Coreper, the committee of the member states' permanent representatives to the European Community.

Jasper Becker

CORRESPONDENCE

Cancer hazards

SIR — Epstein and Swartz (*Nature* **289**, 127; 1981) cited our critique of the report by Bridbord *et al.* on *Estimates of the Fraction of Cancer in the United States Related to Occupational Factors*. Their citation was correct as given, but misleading without our antecedent qualifications. In general, we agree with many others who have reviewed the document that the estimates of occupationally related cancer presented there are probably substantially higher than the data support. In any event, the important question is what reduction in cancer frequency might be achieved by control of occupational exposures. Further epidemiological inquiry should improve those estimates.

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Draize test must go

SIR — The news that Revlon is to finance research into developing an *in vitro* alternative to the Draize test for eye irritancy should be welcomed by everyone, not just by anti-vivisectionists (*Nature* 19 March, p.181). The Draize test is notoriously unreliable, yet it has survived basically unchanged for over 36 years. A replacement is long overdue.

As far back as 1971 a survey of intra- and inter-laboratory variation in the interpretation of results indicated that the Draize test was inadequate for assessing eye irritancy even on a simple pass/fail situation. As a result of the study it was recommended that, failing moves to improve reliability, the test had little relevance and should be excluded in regulations as a toxicity test. Despite this, the Draize test is still the only recognized test for eye irritancy.

On scientific grounds alone, the Draize test should have been replaced years ago.

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IQ put to test

SIR — In his recent *Nature* article¹ N. J. Mackintosh refers to our research demonstrating a relationship between IQ and "inspection time", the latter being a measure assumed to reflect the speed at which a person processes information.

Although we have found inspection time to be correlated with IQ, this relationship becomes statistically significant only for samples with widely varying IQ scores. The correlation coefficient of -0.92 between inspection time and IQ was obtained from a group of 10 individuals whose IQ scores ranged from 47 to 119. We emphasized that this correlation should be interpreted cautiously because of the manner in which the sample was chosen. Furthermore, this relationship was found only with respect to Performance IQ scores, as measured by the Wechsler Adult Intelligence Scale; correlations between Verbal IQ and inspection time estimates were much smaller and not statistically significant². Our study involving 48 subjects, and which found a correlation of -0.80 between Performance IQ

and inspection time³, also included retarded persons. Here again the strong correlation was the consequence of a very wide range in IQ scores. The study involved three groups, each of 16 adolescents; a retarded group where IQ scores ranged from 57 to 81, a group of nonretarded individuals with IQ ranging from 90 to 115, and a third group with scores between 116 and 130. We found no difference in the values of inspection time for the two nonretarded groups (mean = 93 ms, standard deviation = 40 ms and mean = 90 ms, standard deviation = 42 ms). Further analysis of these data (not previously published) confirms only a low correlation between inspection times and IQ for the 32 nonretarded subjects ($r = -0.23$).

At this stage of our research, it is not clear what inspection time is measuring. It is one of a number of measures⁴ developed within a model which attempts to account for capacity limitations, response bias and the caution with which a response is made⁵. The inspection time index suggests that it may prove useful for investigating what limits there are to the speed of human information processing. Insofar as it turns out to be an adequate measure of a sub-process involved in human decision making, it may conceivably be a factor in what might be termed intelligent behaviour, assuming as is widely held that this involves a speed component. To suggest more at this stage, however, seems unwarranted.

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Department of Psychology,
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Why conserve?

SIR — Without seeking to emulate the composer Johann Joseph Fux, who "could not resist being drawn into endless polemics"¹ we feel that some of the points raised by Dr Muir² require critical examination.

The argument that a species or habitat need not be preserved after it has been "competently" investigated raises several questions. First, what criteria can be used to decide whether a particular investigation has, in fact, been competently executed? After the completion of a study by one group of investigators, will it be possible for other workers to confirm or refute their findings, or will the relevant biological phenomenon already have ceased to exist? Second, is it realistic to suppose that everything can be learned about a given phenomenon using presently available experimental techniques? Will future techniques have nothing new to contribute? Third, are biologists concerned only with documenting facts which immediately become historical, rather than remaining current?

Perhaps the most surprising feature of Dr Muir's arguments is that they are utilitarian. Their implication is that a species or habitat should not be permitted to exist for its own

sake, but only if it provides an opportunity for human exploitation. It must be unusual for a professional biologist to express this opinion.

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Lamarck's revival

SIR — The following type of argument has always struck me as unfair to Lamarck and beneath the dignity of a respectable scientist. On the one hand, the emphasis of microevolution and minuteness of changes from one generation to the next, combined with extreme refinement and a plethora of sophisticated tricks (mutation, selection, drift, spatial isolation, genetic isolation, etc.) employed in defence of Darwinism and, on the other, the arrogant vulgarity in discrediting Lamarckism, for example, "why does not a woman whose arm is amputated produce one-armed children?" (ref.1), "can an animal practice being purple?" (ref.2).

The essence of the scientific argument is the validity or otherwise of the Weismann doctrine. It seems logical that resistance to transformation of somatic changes into genetic ones must be high to prevent an organism from the danger of over-reacting to environmental fluctuations and to make it maintain a certain stability. But, in view of the importance of feedback in nature, it seems unlikely that the Weismann barrier is completely leakproof under sustained nonrandom directional stress. Even from the Darwinist viewpoint such complete rigidity and unresponsiveness does not make much sense.

Whether Steel and Gorczynski³ are right or wrong is not so much important in this context. What is extremely important however, is that they are forcing the discussion onto a scientific level which it deserves.

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Evolution on trial

SIR — Following the evolution trial in Sacramento, California, thanks are due to scientists who responded to requests from Mr Tyler and myself, acting as his consultant, to be available as witnesses. These were Drs C.R. Berger, B. Dalrymple, R. Dickerson, R. Doolittle, A. Kornberg, J. Kumamoto, W. Laetsch, R. Lemmon, W. Mayer, E. Olson, C. Sagan and D. Wake, evolutionists all, who generously agreed to undergo, if needed, the inevitable frustrations and delays of courtroom trials.

The next "round" will presumably be in Arkansas, where legislature passed a bill, on 17 March, prescribing the teaching of creationism.

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NEWS AND VIEWS

A tropical Volute shell and the Icarus syndrome

from Jonathan C. Howard

DARWIN tells a little parable in his autobiography¹ which he learned as a very young man, before the *Beagle* voyage, from Adam Sedgwick, the Professor of Geology in Cambridge.

"Whilst examining an old gravel-pit near Shrewsbury, a labourer told me that he had found in it a large worn tropical Volute shell, such as may be seen on the chimney pieces of cottages; and as he would not sell the shell, I was convinced he had really found it in the pit. I told Sedgwick of the fact, and he at once said (no doubt truly) that it must have been thrown away by some one into the pit; but then added, if really embedded there it would be the greatest misfortune to geology as it would overthrow all that we know about the superficial deposits of the Midland Counties. These gravel-beds belong in fact to the glacial period, and in after years I found in them broken Arctic shells. But then I was utterly astonished at Sedgwick not being delighted at so wonderful a fact as a tropical shell being found near the surface in the middle of England."

The point of the parable is that science does not merely consist of making observations but also of ordering them in relation to each other. The tropical shell did not fit into an ordered scheme, and so either the shell or the scheme was wrong. Sedgwick backed the scheme against the shell and won. There would be nothing unscientific about backing the shell, but the quality of support would have to be strong in proportion to the length of the odds against it. No stone could be left unturned to verify every aspect of the find, and eventually a hypothesis would have to be formulated of sufficient generality to accommodate the coexistence of Arctic and tropical shells in the same deposits. Even then the pre-existing scheme would probably not be 'wrong' but merely incomplete. Sedgwick rather overstated the case. New, confirmed and revolutionary discoveries in science very rarely utterly degrade an existing scheme and rebuild its elements in an entirely new pattern. In biology only the theory of evolution by natural selection came close to doing this, and most of the material incorporated into

the new theory was altered only in context rather than form.

The scientific temperament varies, no doubt, along many dimensions. But certainly one of the most interesting is the dimension of credulity. How eccentric does a finding or proposition have to be before it runs off the end of your credulity scale? Are you for the shell or for Sedgwick? Incredulity was my first reaction when I heard by the usual roundabout route that Dr Gorczynski of the Ontario Cancer Institute was reporting the transmission of acquired immunological tolerance through the male. I assumed that the grapevine, with its usual capacity for decreasing the signal-to-noise ratio, had got the sex wrong. Maternal transmission would have fitted comfortably into a well ordered scheme since mother and fetus are known to engage in a rather wide-ranging immunological discourse both before and after parturition; but the grapevine had got the sex right, and here was our tropical Volute shell in the glacial gravel pit. What the grapevine did not say was anything about the genesis of the experiment, and this was a grave omission. Gorczynski's collaboration with Dr E. Steele was conducted with the specific intention of searching for paternal transmission of tolerance, under the influence of Steele's already formulated hypothesis² that RNA viruses might act as vectors for the horizontal transmission of the DNA-coded information from somatic cells to cells of the germ line. According to this hypothesis, transmission through the male is more probable than through the female because male germ cells are formed continuously throughout life.

The phenomenon became public knowledge when the experiments were first published last year³. The level at which I have heard these experiments and their sequel⁴ discussed has varied from gleeful iconoclasm, through critical scepticism tinged with curiosity, to frank disbelief. More upsetting is the charge that the data are suspect because the experiments were

designed and conducted under the influence of a hypothesis of high import. Under these conditions, so the argument runs, the eye is guided more by hope than by the ineluctable evidence of the γ -counter. I assumed that this naïve folly had been killed by Sir Peter Medawar's graceful and persistent advocacy of the importance of hypothesis as a guide to useful scientific action, but under the provocation of Gorczynski and Steele's results it seems to have risen from the grave. Since Darwin is often pushed into the ring to confront Steele, I might add that Darwin unequivocally endorsed Steele's logistical procedures when he wrote⁵: "How odd it is that anyone should not see that all observation must be for or against some view if it is to be of any service".

As for the gleeful iconoclasm, it reminds me of the scornful laughter which greeted my Latin master when he muffed an easy declension. He remained an incomparably better classicist than we were. How much of neo-Darwinism are the iconoclasts prepared to throw out on the strength of Gorczynski and Steele's findings? Weissmann's doctrine of the isolation of the germ plasm from somatic influence was a brilliant hypothesis. If it had been badly wrong, genes would not behave in the elegant and predictable way that they do. In the context of Steele's hypothesis, if viral vectors are engaged in the transmission of DNA between cells, and, as implied by Gorczynski and Steele's recent paper in *Nature*⁴, are no respecters of the linkage group, we should have no loci and no alleles. The chromosomes of the germ cells would be a randomized dump of DNA sequences gathered from cells all over the body (see Darwin's *Provisional Hypothesis of Pangenesis* for a nineteenth century expression of this idea)⁶. Every time alleles behave like alleles and linkage groups behave like linkage groups it is another conformation of the generality of mendelian genetics and of the integrity of the germ line, and a further exception to the generality of Steele's hypothesis. However, since neither Weissmann's doctrine nor mendelian genetics predict Gorczynski and Steele's findings, the obligation falls on everyone concerned to find out what

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kind of exception to the order of things these phenomena are.

It is the mixture of critical scepticism and curiosity alone that is going to resolve this matter. It is a tribute to the vigour of these faculties in the immunological community that this issue of *Nature* carries two manuscripts which go directly to the heart of the scientific problems on hand. First and foremost, are Gorczynski and Steele's results readily reproducible? Brent and colleagues (see p.508) have conducted one of the most serious attempts I have ever seen in immunology to reproduce an experimental protocol in detail. Furthermore, the analysis of the phenomenon is extended in two ways: tolerance was assessed by skin graft rejection as well as the capacity to generate cytotoxic cells *in vitro*, and repeated tests of *in vitro* reactivity were performed on individual animals. There is no escaping the conclusion that, under the conditions of these experiments, the Gorczynski and Steele result has not been confirmed. The explanation for the inconsistency between the two results is now confined to the residual procedural differences between the two series of experiments. Brent and colleagues draw attention to several such differences, only one of which is, in my opinion, important. This difference is in the cell populations used to induce tolerance in the experimental fathers. Gorczynski and Steele's tolerance-inducing protocol was eccentric in the extreme: enormous doses of cells were injected repeatedly into the fathers from birth until the end of the breeding period. Heroically, Brent and his co-workers repeated this anomalous procedure, but they made one mistake. The very first injection of cells, given on the day of birth, consisted of a total of 10^8 composed of equal quantities of bone marrow and spleen cells in Gorczynski and Steele's experiments, and 5×10^7 composed of one part of marrow to ten parts of spleen in Brent and colleagues' experiments. Brent's group is inclined to minimize the significance of this difference, but this seems a foolhardy attitude. Since there was, until Gorczynski and Steele's results were published, no reasonable expectation that any protocol would work, Brent and colleagues cannot dismiss any substantial feature of the successful protocol as insignificant. Curiosity and scepticism are, I am glad to read, carrying these authors still further to the point of reproducing even this aspect of the original protocol in their future experiments.

The importance of this difference in protocol is impossible to assess at present, but there is one crucial point which deserves more emphasis than Brent's group give to it. Amazingly, despite the ruthless régime of tolerance induction, the experimental fathers were apparently not fully tolerant by the measure of their ability to produce *in vitro* cytotoxic activity against the tolerizing parent. In Gorczynski and

Steele's experiments the tolerant fathers were shown to be as tolerant as normal F_1 hybrids. Gorczynski and Steele's *in vitro* induction system was less efficient than that of Brent and colleagues, and weak residual activity may not have been visible, but the difference between apparently fully tolerant and apparently not fully tolerant fathers cannot be considered trivial in an experiment on the transmission of tolerance. Perhaps the difference is real and important, and perhaps it too hinges on the difference in the cellular composition of the first inoculum used to induce tolerance.

When Gorczynski and Steele's first results were published, discussion among colleagues threw up two further kinds of experiment which we felt would explore the generality of the original claim. As self-tolerance is presumably an acquired characteristic, the A homozygous progeny of an $(A \times B)F_1$ father mated to an A mother might show inherited hyporesponsiveness to B alloantigens. Second, since the method of inducing tolerance used by Gorczynski and Steele was so unusual and must have resulted in a high level of chimaerism in the tolerant fathers, we wondered idly what would be the immunological behaviour of the progeny of male $A \leftrightarrow B$ embryo fusion chimaeras with extensive chimaerism in all tissues, ideally including the gonads as well. So rapid and relentless is the march of science that both our lunchtime speculations have already appeared as substantial experimental results. Hasek and colleagues⁷ have done the backcross experiment and McLaren and colleagues (including some of Brent's group) the embryo fusion chimaera experiment (see p.513 in this issue *Nature*). In neither case was any detectable level of specific hyporesponsiveness found in the progeny of the appropriate matings. Again, therefore, Steele's hypothetical mechanism finds itself narrowly constrained by results. If acquired immunological tolerance can be inherited through the male, then the conditions under which this occurs are special and not yet adequately defined. Weissmann and Mendel still provide the general case. We need an explanation for Gorczynski and Steele's tropical Volute shell, but the superficial deposits of the Midland counties are still of glacial origin.

There is much good in this fascinating transaction. A novel and imaginative hypothesis has provoked a series of experiments which would probably never otherwise have been done, certainly not with so much care. The results are conflicting, but there is a *prima facie* case from Gorczynski and Steele's two successful studies for continuing to treat the matter as being of the greatest importance. Even if it turns out, surprisingly, that both sets of positive results have some completely trivial explanation, our perspective will still have been enduringly enriched by the reminder that sex may not be the only way of trans-

ferring DNA between cells.

There is also something bad about what has happened over the past few months, which concerns the fragile and ill articulated matters of tact and style in the presentation of scientific material. Steele's book, *Somatic Selection and Adaptive Evolution*, was unlikely to have turned many heads without some truly original experimental support. It conflates an unacceptably wide variety of experimental findings into a single scheme, and fails in its obligation to apply an intensive critical appraisal in conventional terms to each set of findings in turn. The issue with which it deals, the mechanism of adaptive evolution, is not an entirely open question. Recent evidence for great complexity in the organization of genetic material shows that the beguiling simplicities of the double helix and the genetic code are probably not sufficient to explain all the properties of evolving systems; but this does not mean that all that has gone before can be discarded. Natural selection remains in the pre-eminent position that Darwin and Wallace created for it — as a mechanism for adaptive evolution. One of the most important subsidiary conclusions of genetical research in the last half-century is the recognition that developmental plasticity or individual adaptability to environmental heterogeneity is an evolved character under genetic control. C.H. Waddington⁸ pointed out as long ago as 1942 that genetic modifications to adaptable systems can stimulate the inheritance of acquired characters. In relegating discussion of this fundamentally important concept to a dismissive footnote, Steele does both himself and modern genetics a disservice. It is the current fashion to level popular attacks at the theory of evolution. Twice in the past year Darwin could be found staring gloomily from one corner of the *Sunday Times Magazine*, whilst first Lamarck and then Professor Stephen Gould of Harvard University confronted him from the opposite corner. Whether Professor Gould would wish to represent the opposition to classical Darwinism seems doubtful, but the earlier confrontation with Lamarck was designed to represent the challenge raised by Steele's work. If Steele is feeling the heat of an intensive critical scrutiny, both of his ideas and of his experiments, it is because, like Icarus, he has flown close to the Sun. □

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Small is beautiful — portrait of a mitochondrial genome

from Piet Borst and Leslie A. Grivell

PUBLICATION of the complete sequence of the 16,569-base pair human mitochondrial DNA (mtDNA) on p.457 of this issue of *Nature* is a landmark in biology. It comes only 15 years after mtDNA from vertebrates was found to consist of rather small DNA circles¹⁻³, and only 6 years after Sanger and his co-workers developed the sequencing methodology to tackle such large molecules. Together with the virtuoso analysis of mitochondrial transcripts by Attardi's group, reported on pages 465 and 470 of this issue of *Nature*, the sequence provides not only a wealth of information on mitochondrial biogenesis, but also insights into the genetic code and decoding, and into what it takes to make a ribosome, to read messenger RNA (mRNA) and on the minimal requirements for transfer RNA (tRNA) function.

The organization of information in human mtDNA shows an economy never before encountered in nature. Not only does the sequence pack the genes for 2 ribosomal RNAs (rRNAs), 22 tRNAs and 13 assorted proteins so tightly that there are few or no non-coding bases between them, but some of them have been stripped of features that have come to be regarded as *de rigueur* for a respectable gene. In particular, the mRNAs lack non-translated

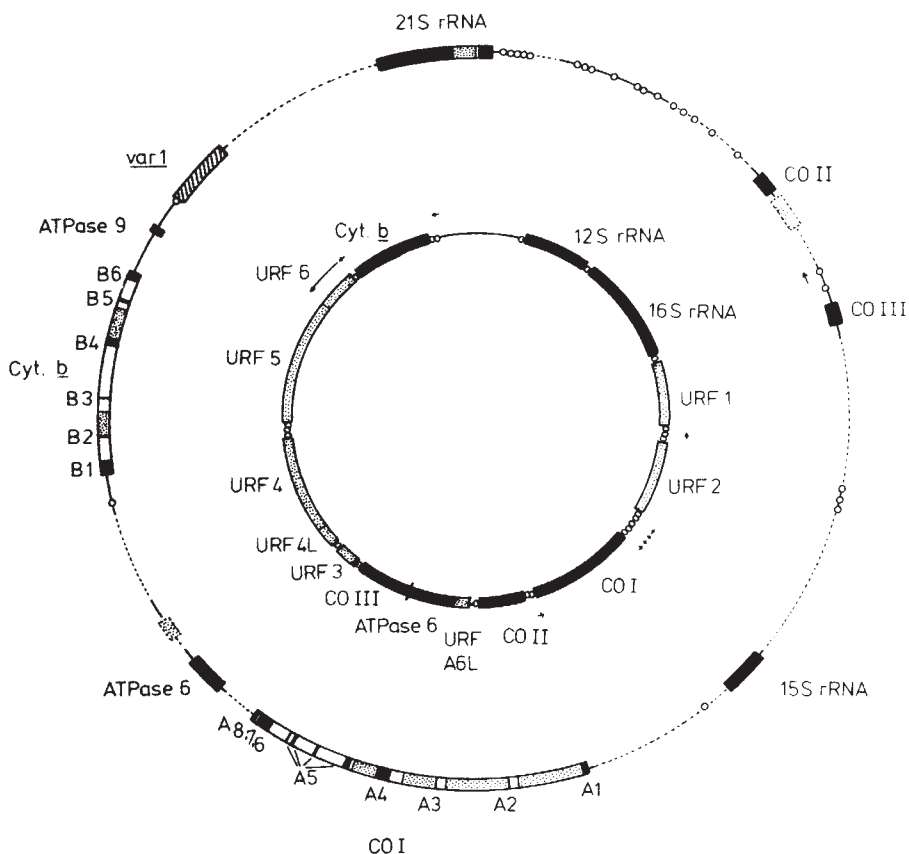
leader and trailing regions. Furthermore, over half of them do not even have a complete stop codon; this only materializes on polyadenylation of the 3' terminus, whereby the terminal U or UA of the gene is converted to the stop codon UAA. Even the transcription-translation machinery used for the expression of human mitochondrial genes has not escaped the drive for economy; features familiar in the genomes of all organisms from viruses to man have been thrown overboard. Thus, transcriptional regulation is a primitive affair, with only one promoter for RNA synthesis on each strand. Consequently, the genome is completely and symmetrically transcribed. Differential gene expression is apparently achieved by a combination of transcription-attenuation, control of the cleavages required to generate transcripts of individual genes and stability of the final transcripts. The rRNAs have been reduced to a size (1,559 and 954 nucleotides) not found outside mitochondria; a 5S RNA is absent and only 22 tRNAs, of highly unorthodox structure, are used to read all codons. This remarkable feat is accomplished by having some tRNAs read four codons and in these, U is in the first position of the anticodon, permitting U:N wobble.

An important spin-off of the sequence analysis is the finding that the genetic code in human mitochondria differs from that used in other genetic systems: UGA is read as tryptophan rather than 'stop', AGA and AGG as 'stop' rather than arginine, AUA as methionine rather than isoleucine, and AUA or AUU is sometimes used as an initiation codon instead of AUG. The genetic code is therefore not universal.

For the mitochondriacs who spend their days studying mitochondrial biogenesis, the human mtDNA sequence also has an interesting bonus. In addition to the genes for subunits of major membrane proteins like the cytochrome *c* oxidase, apocytochrome *b* and the ATPase complex, which were already known to reside in mtDNA, sequence analysis has unearthed eight URFs (unassigned reading frames, coding for as yet unidentified proteins). There is very little doubt that these sequences represent protein genes because they are conserved in the bovine mtDNA and, moreover, it is highly improbable that such a tightly packed genome would contain non-functional genes. The function of the

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Fig. 1 Organization of yeast and human mitochondrial genomes. The figure summarizes our present knowledge about the organization of genes on human mtDNA and the yeast *Saccharomyces cerevisiae* strain KL14-4A (~78,000 base pairs). For clarity and to accommodate both genomes within the same format, the human genome has been drawn approximately 2 times 'larger-than-life' relative to yeast. On both DNAs, sequences encoding known gene products are shown as black bars, URFs are indicated by stippled areas and open circles represent a tRNA gene. Transcription is in a clockwise direction, unless otherwise indicated. The cross-hatched region in the yeast genome shows the location of the *var1* genetic determinant, which is involved in the specification of a mito-ribosomal-associated protein. The exact localizations of the DNA sequences encoding this protein are as yet unknown. For yeast, the figure is based on DNA sequence data from several laboratories¹⁸⁻²³, supplemented with unpublished data from our own laboratory (L.A.M. Hensgens, personal communication). Sequenced areas are denoted by a continuous line, unsequenced areas by a broken line. The inventory of genes is thus a provisional one. In the split genes for apocytochrome *b* and COI introns not containing an URF are shown as open bars; exon nomenclature is from refs 19 and 23. The URF in the intron between B2 and B3 in the cytochrome *b* gene has been identified as the C-terminal half of an RNA processing enzyme or maturase (see refs 19 and 24). COI, II, III are subunits of cytochrome *c* oxidase, *cyt. b* is apocytochrome *b*, ATPase 6 and 9 are subunits 6 and 9 of the ATPase complex. URFs are unassigned reading frames. The arrows indicate genes transcribed from the strand opposite the one that encodes most genes.



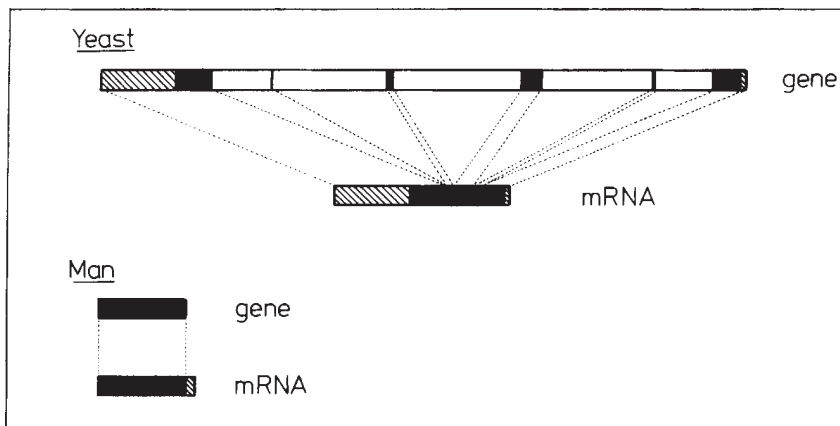


Fig. 2 Comparison of the gene and mRNA for apocytochrome *b* in yeast (see ref.24) and man (refs 4, 5 and S. Anderson *et al.* p.457 of this issue of *Nature*). Coding sequences are shown as filled-in bars and introns as open bars.

proteins corresponding to the URFs remains a matter of speculation (RNA processing? Ribosomal proteins?).

How representative of mitochondrial genetic systems is the human's? The size of mtDNAs from sea urchins, flatworms, insects and man is similar⁴ and the relative positions of tRNAs and other transcripts on human and toad mtDNA is virtually the same⁵. But an entirely different situation is found in yeast, which contains a fivefold larger mtDNA. Figure 1 compares the overall gene order in the two mtDNAs. Yeast contains a series of URFs showing no significant homology with their human counterparts, but has a set of genes for major membrane proteins which are about 50 per cent homologous to their human mitochondrial equivalents. The order and the space in which these genes are arranged in the DNA are, however, completely different. The striking difference in compactness is exemplified by the comparison of the apocytochrome *b* genes, presented in Fig. 2. The coding sequences are almost identical in size, but the yeast mRNA has a leader sequence of 1,000 nucleotides, whereas the human mRNA has none. The yeast gene is split by five introns, the human gene (like all other human mitochondrial genes) is not. The yeast gene has a non-translated 3'-trailing region of about 50 nucleotides; the human gene has no such region and even lacks a complete stop codon. The human mRNA is polyadenylated; the yeast mRNA seems not to be. Obviously there is no pressure for maximal economy on yeast mtDNA. Further evidence of that comes from the long non-coding stretches between genes.

Although RNA processing is prominent in both human and yeast mitochondria, in yeast transcription appears to start from at least five separate promoters⁶ and tRNA punctuation, so elegantly used by human mitochondria in RNA processing (see p.470-475 of this issue), does not have a role. With less pressure for economy one would expect the yeast rRNAs and tRNAs to be less 'petite' and unusual than their human counterparts. This is the case; the rRNAs in yeast mitochondria are about 3,200 (ref.7) and 1,660 (ref.9) nucleotides long and the tRNAs, although lacking some of the common features of tRNAs used in

non-mitochondrial genetic systems^{8,11}, are less bizarre than the human ones. As in the human, the yeast mitochondria have some tRNAs able to read four codons but they contain more iso-accepting tRNAs, the total gene number now being at least 25, against 22 in human mtDNA. Finally, yeast, like human, mitochondria read the UGA codon as tryptophan rather than 'stop', but they do not share with human mitochondria the unusual assignments of the AUA, AGA and AGG codons. Instead they read the CTN codon as leucine rather than threonine¹², so there is not even a common code in different mitochondria.

Why does human and animal mtDNA look like a 1981 university department, with everything reduced to minimal size without anyone actually being fired, whereas an equivalent set of genes is spread luxuriously over 78,000 base pairs in yeast? We think that the key to an understanding of these differences lies in the rapid evolution of the mtDNA genes that code for rRNAs and tRNAs. For instance, the large rRNA from mouse¹³ and rat (C. Saccone, personal communication) mitochondria share only 68 per cent homology. (Compare this with the 74 per cent homology between the 16S rRNAs of *Zea mays* chloroplasts and *Escherichia coli*¹⁴.) And, at most, human and beef tRNA^{thr} are only 74 per cent homologous. Furthermore, the T ψ C loop, which has a constant seven-base length outside mitochondria, is three bases long in human and eight in bovine tRNA^{thr} (ref.15).

In our opinion this high evolutionary divergence of rRNA and tRNA genes in the mitochondria of closely related species is most easily explained by the low demands made on the mitochondrial translation system. This system has only to produce a very limited number of proteins and usually at a constant relative rate. It may therefore evolve more rapidly than a system which has to produce a large number of proteins at maximal efficiency and subject to various translational controls. Different selective pressures must have pushed animal mtDNA into extreme economy but yeast into maximizing the A+T content. The nature of these pressures is unknown.

If the mitochondrial genetic system can

take more change than other systems, it is possible that further searches in the backstreets of biology may uncover additional surprises. The small mitochondrial genomes of, for example, *Chlamydomonas*, with 15,000 base pairs¹⁶, or *Trypanosoma brucei*, with 20,000 base pairs¹⁷, exhibit different or more extreme solutions to the pressure for economy than human mitochondria. Indeed, the trypanosomes appear to have even smaller rRNAs in their mitochondria (1,230 and 640 nucleotides) than man¹⁷. The study of such extremes may deepen our insight into the minimal requirements for a functional genetic system and provide clues about the evolutionary pathways that might have been followed to develop the more sophisticated mechanisms of gene expression used by present-day bacteria and eukaryotes.

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Thrombosis: a molecular approach to therapy

from P.J. Gaffney

VENOUS THROMBOSIS is thought to be initiated by the thrombin-mediated conversion of circulating fibrinogen to fibrin and the deposition of long fibrin polymers on the vessel wall, with the simultaneous aggregation of platelets and entrapment of other cellular components of blood. There is evidence that fibrin deposition is also involved in the progressive growth of atheromatous lesions, which characterize coronary artery disease. While present-day pharmacological approaches to the alleviation of occlusive vascular disease frequently fail to solubilize the target fibrin without hazardous systemic effects, in this issue of *Nature* (p.505) Smith and his colleagues describe a novel chemical tactic using chemically modified enzymes and activators, which allows the delivery of a fibrinolytic action direct to the target fibrin, leaving the circulating components of the haemostatic system unaffected. Current views about the molecular reactions involved in fibrin formation and destruction allow a better appreciation of the ingenuity of this new approach to thrombolytic therapy.

The generation of thrombin is a central event in the initiation of a thrombus in that this enzyme (1) causes platelets to aggregate, (2) activates fibrinogen to fibrin, and (3) converts an inactive plasma transglutaminase (Factor XIII) to its active form. The latter two reactions are involved in the establishment of an organized insoluble fibrin network which 'cements' the various components of a thrombus together. The interaction of thrombin with fibrinogen involves the removal of small peptides from the dimeric fibrinogen molecule¹, exposing a previously masked dimeric polymerization site on the fibrin monomer² which can attract two fibrinogen molecules in a polymerizing reaction. These latter fibrinogen molecules are converted to fibrin by the action of thrombin, and further accumulation of fibrinogen feeds the multi-branched growth of the fibrin polymer network. Activated Factor XIII catalyses the formation of stable glutamyl-lysyl cross-links³ between the chains of the soluble forming fibrin and this cross-linking process is completed in the insoluble fibrin found in most established thrombi⁴. Thus the *in vivo* formation of fibrin clots is a dynamic process with all the events in a series of reactions being interdependent.

Of necessity there are a number of control mechanisms on clot formation. While inhibitors of thrombin (particularly ATIII) are crucial in the control of thrombin generation, it is generally agreed that the fibrinolytic system protects against fibrin deposition and thus against most

forms of thrombosis. The fibrinolytic system consists of a circulating proenzyme (plasminogen) and a plasma activator (plasminogen activator), the latter being stored in and released on demand from the endothelial cells lining the vessel wall. The interaction of plasminogen activator with plasminogen produces the active serine protease, plasmin, which is very rapidly neutralized by natural inhibitors in the plasma, notably α_2 -antiplasmin⁵. The role of the fibrinolytic system in controlling fibrin generation is best exemplified during disseminated intravascular coagulation, which is a serious complication in a variety of seemingly unrelated conditions (such as cancer, pregnancy and septicaemia). During this form of intravascular coagulation the systemic generation of thrombin is accompanied by the activation of the fibrinolytic system which digests the forming fibrin before it can act as a focus of local thrombus formation on the vessel wall. It seems that as fibrin forms, it orchestrates its own destruction; some of the mechanisms of this orchestration process are now understood (for review see ref.6).

Plasminogen activator and plasminogen bind strongly to forming fibrin while the major fibrinolytic inhibitor, α_2 -antiplasmin, has no such affinity. Thus, this inhibitor, which has such a powerful and immediate neutralizing effect on free plasmin, can only mildly affect plasmin attached to forming fibrin. The preferential binding of plasminogen to fibrin is via a lysine-binding site on the A chain of the plasminogen molecule, which is distinct from that region of the molecule where the catalytic site resides. This lysine-binding site and the active site of plasmin both combine with α_2 -antiplasmin during the rapid neutralization of plasmin. Thus, the protective effect of fibrinolysis against thrombosis seems to be regulated by the localization of plasminogen activator-plasminogen complex on, and the exclusion of inhibitors from, the surface of the forming fibrin⁷.

It is reasonable to propose that the mechanisms described above for systemic intravascular coagulation also apply during the localized coagulation which precedes the occurrence of thrombosis. Should the fibrinolytic system be unable to control such coagulation episodes, it probably cannot digest the consequent organized thrombi containing resistant cross-linked fibrin. There is, indeed, a developing consensus that enzymes from entrapped cells are responsible for the

spontaneous lysis of thrombi⁸. This non-plasmin form of fibrinolysis probably proceeds quite slowly, whereas urgent and effective thrombolytic therapy is required during life-threatening thrombosis, which involves imminent tissue death in vital organs.

The major aim of treatment must be the destruction of the target fibrin in the thrombus, while leaving unaffected the fluid components of the blood. Though two potent fibrinolytic agents, streptokinase (SK) and urokinase (UK), have been in clinical use for the past 20 years, wholly satisfactory therapy has not yet been developed. Urokinase directly converts plasminogen to plasmin while SK forms an equimolar complex with plasminogen or plasmin (SK-plasminogen or SK-plasmin) and this complex, like UK, converts plasminogen to plasmin⁹. While the systemic plasmin so formed is rapidly neutralized by α_2 -antiplasmin, any plasmin generated in excess of the neutralizing effect of α_2 -antiplasmin arguably attacks not only the occlusive thrombus, but also circulating fibrinogen and other components of the haemostatic process. Since these effects are systemic and the thrombus may be localized at one point in the circulation, it is not surprising that both the destruction of fibrinogen and the consumption of α_2 -antiplasmin occur. Damaged circulating fibrinogen and its effect on platelet aggregation have been proposed as major causes of the haemorrhages sometimes occurring during infusion of SK and UK for the relief of occlusive vascular disease¹⁰.

Smith and his colleagues, in their novel approach to the therapy of venous thrombosis, exploited the differential affinities of plasmin for fibrin and α_2 -antiplasmin and demonstrated that acylation of the active site serine allowed plasmin to bind exclusively to fibrin without being inhibited by α_2 -antiplasmin. Likewise, an equimolar SK-plasmin activator complex was acylated and, when infused systemically in experimental animal models, it bound to thrombi without activating the circulating plasminogen. The authors demonstrated the *in vitro* deacylation of plasmin and activator while bound to fibrin and their results in animals indicated a similar deacylation process on the surface of experimental thrombi, causing effective lysis. During these experiments little or no systemic side effects were observed.

This approach provides an attractive alternative to the somewhat hazardous systemic approaches used to date in thrombolytic practice, which are quite often accompanied by the destruction of some essential haemostatic components of

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blood with accompanying danger of haemorrhage. The successful targeting of the fibrinolytic action to the fibrin in experimentally induced clots in rabbits and dogs, without overt systemic effects, opens up a new approach to thrombolytic therapy. However, before undue optimism is generated, there is a need to demonstrate these different thrombolytic and systemic effects using acylated enzymes and activators in a whole human system (that is, in a system where a human thrombus is perfused with human blood). The efficacy of this new approach must also be tested in other models of thrombosis which are more

closely associated with the vessel wall. Should these approaches be successful, then a new dimension in the treatment of thrombotic disease may soon be available for testing in clinical trials. □

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Stable clocks and the theory of relativity

from G.J. Edwards

EINSTEIN'S theory of relativity is to my mind the most elegant of all scientific theories. Based on the simple hypothesis that all local, freely falling, non-rotating laboratories are fully equivalent for the performance of all physical experiments (the principle of equivalence), the theory has vast ramifications which extend throughout physics and form a basis for our understanding of the structure of the Universe. The theory has, of course, been something of a theoretician's paradise and an experimentalist's hell, but the past twenty years have seen two developments which somewhat redress this imbalance. These are the ability to hurl sensitive equipment into space and the great improvements of oscillators (clocks) such as masers, lasers and atomic clocks, some of which now have stabilities of 1 part in 10^{14} or so over significant periods of time. The kind of accuracy possible using these aids is exemplified by a recent experiment by Vessot and colleagues (*Phys. Rev. Lett.* 45, 26, 2081; 1980) which is the most precise confirmation to date of the change in the rate of a clock moving in a gravitational field. The experiment also points the way for future projects which will probe accurately the actual structure of space and time.

In the above experiment (carried out by the Smithsonian Astrophysical Observatory and the George C. Marshall Space Flight Center, US) a very stable clock was launched by rocket to a height of 10,000 km in a near-vertical trajectory. The clock was a hydrogen maser operating at 1,400 MHz and with a stability of 1 in 10^{14} over 100 seconds. After the 18-g launch acceleration, the spacecraft containing the maser went into free fall, allowing about 100 minutes of experimental time, during which the frequency shift in the maser

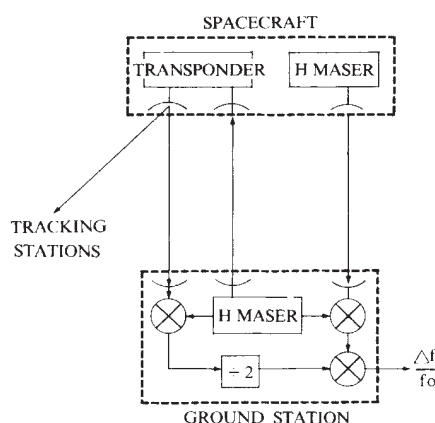


Fig. 1: Simplified schematic diagram of the experiment by Vessot *et al.*

signal radiated to Earth was measured and, after correction, compared with the value predicted by relativity theory (Fig.1). The main correction was in fact large, being caused by the non-relativistic Doppler shift in radiation from a moving source. Its magnitude was obtained using a separate two-way transponder system between Earth and the spacecraft, as the returning signal contains essentially only the Doppler shift. Finally, the position and velocity of the spacecraft need to be known accurately, and these parameters were deduced from the Doppler — shifted signals received at five tracking stations.

The residual fractional shift, $\Delta f/f_0$ in the maser signal is, according to the theory of relativity, given to first order by:

$$\frac{\Delta f}{f_0} = \frac{1}{c^2} [\phi_s - \phi_e - \frac{1}{2} |V_e - V_s|^2 - r \cdot a]$$

The first term is the gravitational red-shift (ϕ_e and ϕ_s are the Newtonian gravitational potentials on Earth and at the maser), while the second term involves the relative Earth-spacecraft velocity and is the famous time-dilation effect for a moving

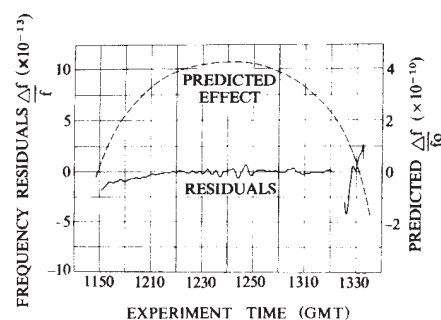


Fig. 2: Frequency residuals and predicted effect during mission.

clock of special relativity. The last term is a residual Doppler shift due to Earth acceleration.

The result of this difficult experiment is indicated in Fig.2. Clearly the experiment was a great success and the authors quote the overall uncertainty as given by:-

$$\frac{\Delta f}{f_0}(\text{experiment}) = \frac{\Delta f}{f_0}(\text{theory}) [1 + (2.5 \pm 70) \cdot 10^{-6}]$$

The previous best direct verification of the velocity-dependent term is from a measurement of the lifetime of fast-moving muons by Bailey *et al.* (*Nature* 218, 301; 1977) with an accuracy of 1 in 10^3 . The gravitational shift had previously been confirmed at the 1 per cent level by Pound *et al.* (*Phys. Rev.* 140, 3B, B788; 1965) using the Mossbauer effect.

Clearly the new result is a significant boost for the theory of relativity and the same is true of other, recent experiments using very stable oscillators. In one of these, Brillet and Hall (*Phys. Rev. Lett.* 42, 9, 549; 1979) rotated a helium-neon laser working at a wavelength of $3.39 \mu\text{m}$ and locked to a cavity, to check for small anisotropies in space, due for instance to the Earth's motion with respect to the Sun or the cosmic background radiation. They could find no anisotropy at the 1 in 10^{15} level, a 4,000-fold improvement over the best previous attempt. Another experiment by Hafele and Keating (*Science* 177, 166; 1972) involved flying four very stable caesium clocks around the world in aeroplanes. This experiment is conceptually identical to that of Vessot *et al.*, but less precise. Again, theory and experiment were in agreement.

The experiments outlined above are important indications of the results which will almost certainly be obtained by the use of very stable clocks within the next few years. Besides the general theory of relativity, there are a number of other theories of gravitation which predict different values for the space-time 'curvature' in gravitational fields. The theories differ in their predictions by very small terms which are second order compared with the components of the equation above. Some of these terms have already been measured to modest accuracy, for example by observations on the advance in

the perihelion of the orbit of Mercury and the bending of light rays round the Sun. (For a general review of relativistic, gravitational experiments see Rudenko *Sov. Phys. Usp.* **21**, 11, 893; 1978). However, tests using stable clocks should be able to advance significantly our knowledge in this area.

One such ambitious venture is the solar probe (European Space Agency) which will fly, amongst other things, clocks very close to the Sun, where the gravitational field is very much greater than on Earth. Besides obtaining valuable information on the Sun's structure, the experiment should enable us to distinguish accurately between several of the competing theories. Another experiment proposed by Will (*Metrologia*

13, 95; 1977) is the comparison of two different types of very stable clocks on Earth. Certain (metric) theories of gravitation predict that all clocks, irrespective of their construction, have identical gravitational redshifts while another class of (non-metric) theories predicts small shifts between different types of clock. Such an experiment would require clock stabilities in excess of 1 part in 10^{15} .

All of these tests impose extremely strict requirements on the stability of clocks and great care in the design of the experiments. The reward might be that we discover tiny but significant effects which would imply a change in our idea of gravity as significant as the difference between the Newtonian and Einsteinian theories. □

this process operating in close binary star systems in our galaxy. But at the centre of quasars things are likely to be less simple, the infall of material will probably be more chaotic for example. Before the radiation generated deep down at the centres of quasars is detected by our instruments it will have interacted, that is been scattering absorbed and re-emitted, by material within the quasar. These effects modify the observed continuum spectrum so a simple power law fit that might arise from synchrotron radiation does not usually provide good agreement with the observations. The multifrequency observations now possible using satellites and ground based telescopes have proved very useful, and the various lumps and bumps by which their spectra deviate from a smooth distribution are now the subject of much research.

Another problem which has attracted much interest since the launch of the Einstein X-ray satellite, is the contribution made by quasars to the X-ray background. The first X-ray results lead to the embarrassing conclusion that if the X-ray emission from all such objects were summed out to large distances it would more than account for the observed diffuse X-ray background in the range 1–3 keV. Of the many assumptions which went into this calculation at least one now appears to have been unjustified. The original sample was biased towards radio loud quasars and the contribution of all quasars was based on the assumption that the majority, which are radio quiet, had similar X-ray luminosities. A larger sample of X-ray quasars shows that this is not so. Radio loud quasars tend to be stronger X-ray sources than the radio silent majority. We now believe that quasars contribute somewhere around 40 per cent of soft X-ray background.

A consensus seems to have been reached on a basic model of the line spectra of quasars, both emission and absorption, although it should be borne in mind that the following numbers are order of magnitude at best. In a typical quasar the emission lines arise in a region about 1 parsec in diameter and this volume contains about 100 solar masses of ionized gas. The distribution of the gas is far from uniform and consists of a large number of dense clouds (perhaps 10^{12} of density 10^9 particles per c.c.). These clouds may not be fully ionized and could contain an unknown amount of neutral gas. The cloud model provides a simple approximation and no information is available on the shapes and distribution around the nucleus. It seems that the dominant source of ionization is from the central source and not from collisions between the clouds, although this process is known to produce the emission lines in some nearby active galaxies. One important and recently recognized feature of the ionization

Quasars: what and where?

from Martin Ward

RECENTLY the Royal Astronomical Society was host to a discussion meeting* on Quasars which, although short, did provide an insight into the astronomical communities' current views on the nature of quasars. The talks appeared to divide naturally into two categories — concerning what quasars are and concerning where they are.

The latter question relies heavily on statistical arguments, often a weak point amongst astronomers. A study of pairs of quasars with very similar redshifts shows that, assuming the redshift is a result of the expansion of the Universe, their projected separations in space are between 5 and 30 million parsecs. As clusters of galaxies are thought to be components of 'superclusters' with dimensions of comparable range the observations may suggest that quasars are in superclusters too faint to be seen.

A minority view is that the origin of the high redshifts, at least in some cases, is not due to the Hubble flow, and hence cannot be used in the standard way to determine the distance of the quasar. Rather, it is claimed that some component of the redshift is an intrinsic property of the quasar itself. Supporting evidence comes from the incidence of high redshift quasars near to low redshift bright galaxies — an unlikely state of affairs, advocates of the non-cosmological redshift would say, unless the quasars and bright galaxies are in some way related, by ejection of the quasars from the bright galaxies for example. A related problem is of quasar alignments (see *News and Views*, **289**; 533, 1981). An impressive number of pairs, and some multiple quasars have been found near to galaxies. The crucial question is whether these distributions are projection

effects, and hence occur by chance, or imply a genuine physical association. The majority of astronomers still doubt a physical association hypothesis because of the *a posteriori* statistics used, and also because no accepted theoretical framework exists to explain the large intrinsic redshifts. In order to calculate accurately the chances of finding a quasar near to a bright galaxy by projection effects, one needs to know the surface density of quasars. This number is very uncertain at faint magnitudes and different assumed densities will change radically the likelihood of a chance quasar/galaxy pair. So the question remains open, but it is unlikely that the majority view will change without evidence of physical association — as might be provided by an unambiguous detection of connecting material for example. Talks given during the rest of the meeting, addressed the question of what quasars are, and it was assumed without comment that quasars are distant and that redshift is a distance indicator.

The continuum radiation from a large number of quasars has now been observed from X-ray to radio frequencies. Variations in the intensity of this radiation over short timescales, in many cases days, strongly suggests an origin in the very central regions of the object. If quasars are assumed to be at great distances it is difficult to see what sort of powerhouse is capable of generating these enormous energies. A general picture which has gained acceptance over the last few years requires the presence, at the center of quasars and related objects, of a super-massive object (black hole) equivalent to something like 10^8 to 10^{10} solar masses. Material, possibly debris from stars torn apart by tidal forces, then falls onto the central object releasing energy as it does so. There is good evidence for a similar, although very much scaled down, version of

*The meeting was held in London on 13th February 1981 and was organised by M. G. Smith and R. F. Carswell.

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process is the role played by soft X-rays which penetrate deep into the gas clouds. By considering this effect there is better agreement between the observed and the theoretically predicted emission line ratios. There is disagreement over whether obscuration by dust is important. This effect reduces the intensity of short wavelength emission lines relative to longer wavelength ones. It is certainly true in galaxies that where there's gas there's dust, but the relative amounts in quasar emission line clouds is at present unknown.

Finally the absorption line problem. It has been known for some time that the broad emission line Lyman alpha often has, superimposed on its profile, numerous narrow absorption lines which are usually blue-shifted with respect to the quasar. This is the so-called Lyman alpha 'forest'. The

question is, do these absorptions arise in material near to the quasar or elsewhere along the long line of sight between us and it? Opinion now favours the latter interpretation. An explanation in which the absorption occurs in the halos of galaxies is an interesting possibility. If this is correct we could use the light from background quasars to study the properties and statistics of galaxies far too distant to be detected by their own feeble light.

It would be wrong to give the impression that a revolution has taken place in our knowledge of what quasars are over the last few years, but neither is pessimism justified. The new multi-frequency continua and high resolution spectral line observations, together with variation studies have improved our understanding. Progress is being made. □

Triggering of volcanic eruptions by Earth tides

from R.S.J. Sparks

A recent study of Kilauea and Mauna Loa volcanoes in Hawaii (D. Dzurisin *Geophysical Research Letters* 7, 925; 1980) shows a weak but significant correlation of the Earth tide maxima with eruptions of Kilauea. No correlation at all was seen with the activity of Mauna Loa — a result which serves to emphasize how individual the behaviour pattern of very similar volcanoes can be.

The stress changes induced in the Earth's crust by fortnightly Earth tides are very small (maximum of 10^{-2} bar) compared with the levels of tectonic stress ($10^1 - 10^3$ bar) and the probable stresses required to fracture a volcanic edifice and trigger eruptions. Although many studies have attempted to link earthquakes with tidal stressing, the results have generally proved negative or inconclusive. One exception was a study of after-shocks by Ryall and others (*Bull. seism. Soc. Am.* 58, 215; 1968) where a good correlation with fortnightly Earth tides was demonstrated.

In a volcanic eruption, pressure builds up over periods of hours to months due to the ascent of lava into the volcano, the exsolution of volatiles or the entrapment of ground water. When the pressure on the volcanic edifice exceeds the strength of the rock, fracturing occurs and allows lava and gas to reach the surface. Fracture strengths of rock in tensile failure are probably in the range 10 to 100 bar, again very much larger than tidal stresses. Thus, at first sight, a correlation with Earth tides

seems unlikely given many other factors which will control the formation of suitable fractures.

Mauk and Johnston (*J. geophys. Res.* 78, 3356; 1973), however, have pointed out that it is the rate of stress accumulation that can be important. Fortnightly Earth tides can have stress accumulation rates of up to 10^{-5} bar s^{-1} . In volcanoes such as Kilauea the stress accumulation rates by increase in fluid pressure within the magma reservoir are as low as 2×10^{-7} bar s^{-1} . Thus, if stress is slowly building up Earth tides could trigger the eruption. On the other hand, stress accumulations of 5×10^{-5} to 8×10^{-4} bar s^{-1} , greatly exceeding the tidal rates, have been recorded from volcanoes in New Zealand.

The study by Dzurisin examines the two volcanoes, Kilauea and Mauna Loa, for which more data are available than any other volcano. The correlation between Earth tides and Kilauean eruptions is reasonably convincing — the probability of an eruption during a week centred on tidal maximum is nearly twice as much as the probability of an eruption in a week centred on tidal minimum. For Mauna Loa, however, there is no correlation. An important lesson is that it is often very dangerous to transfer the results from one volcano to another. Volcanologists should not use Earth tides in general predictions until they know the behaviour of their particular volcano very well. Most of the world's dangerous volcanoes, such as Mount St Helens, simply do not have a long enough record to find out whether there is a correlation. □

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100 years ago

THE ST. PETERSBURG DYNAMITE MINE

The following account of the bomb recently discovered in St. Petersburg, extracted from Russian sources, gives a remarkable picture of the state of society in the empire, where able chemists and expert miners can be found to engage in such desperate undertakings.

It appears from a sketch-plan which accompanied the translation put into our hand, that the mine extended from one side of Malaya Sadobaya Street to the centre of the roadway; the total length of the mine gallery being fifteen paces, the street must be thirty paces, say seventy-five feet wide.

The gallery terminated in a chamber about double its diameter, and in this was found the charge contained in a case twenty-two inches long and eight inches diameter, weighing sixty-five pounds, and beside this a glass jar contained about thirty pounds more of the explosive substance, apparently an excess quantity over that required for the actual explosion. The explosive consisted of a species of dynamite made by mixing nitro-glycerine with powdered charcoal. The description of the fuse, as contained in the Russian account, is very obscure, but would appear to have consisted of a wide heavy glass tube containing an explosive, described some time back in *Nature*, and prepared by mixing nitro-glycerine with about 10 per cent. of gun-cotton, the result being a very explosive substance of a partially gelatinous character. In the midst of this, and surrounded by a mixture of potassic chlorate and antimonious sulphide, was a sealed glass tube containing concentrated sulphuric acid and a leaden weight. The whole was then apparently connected with the dynamite in the case by means of an india-rubber tube also containing explosives. If this was the actual construction the *modus operandi* of the conspirators was very simple, for the heavy glass tube had only to be allowed to fall, when the lead would have broken the sulphuric acid tube, and the chlorate mixture would have at once inflamed, and the explosion of the jelly would have communicated by means of the rubber tube with the torpedo. At the same time it is very difficult to imagine the reasons which could have induced the conspirators to adopt so crude a method when, as it appears from the account, they had at their disposal in the room adjoining that from which the mine was driven no less than four galvanic batteries, with which the explosion could have easily been instantaneously effected by those on the watch for the passage of the intended victims, a method of operation much more consonant with the skilled character of the other parts of the work.

Whatever may have been the real mode intended to have been used by the conspirators, the results would have been sufficiently frightful, as it is probable that the charge found would have made a "crater" of about fifty feet in diameter.

Some of our readers may be glad to learn that the French Association has resolved to curtail the number of scientific meetings in order to extend the time left for excursions and festivals. The programme includes two receptions by the Mayor of Algiers, one by the Governor-General, and a large Arabian *fête* called *Bita*, dancing and singing by native women, &c. Possibly the reported massacre of the Flatters Expedition may put a stop to these ultra-scientific festivities.

From *Nature* 23, 7 April, 531, 542, 1881.

Erratum

In *Monitoring Mount St Helens* from M. McCormick (*News and Views* March 12, 290, 88, 1981) the value of aerosol mass added to the background stratosphere calculated from lidar data was incorrectly printed as being 0.4 to 0.6×10^{10} metric tons. The value should have been 0.4 to 0.6×10^6 metric tons (p.88, line 46) which agrees with that calculated from SAGE data. The predicted warming of the stratosphere near the peak aerosol concentration at 18km (p.88, line 62) was also incorrectly printed as 'a degree' instead of 'tenths of a degree'.

REVIEW ARTICLE

Membrane protein oligomeric structure and transport function

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Proteins which traverse membranes tend to have a dimeric structure in which the dimer is arranged asymmetrically across the membrane with the axis of symmetry perpendicular to the membrane plane. This general structure is well suited to the function of transporting nutrients across the cell membrane.

THERE is growing evidence that membrane proteins exist in oligomeric (usually dimeric) form, based on studies both of the intact membrane and of solubilized proteins. In reviewing the evidence here, I shall consider only those proteins which clearly or probably traverse the cell membrane. It is my aim to show that the structural features of these proteins have functional consequences which are compatible with the experimentally established prerequisites for carrier systems.

Examples of oligomeric membrane proteins

Studies of the intact membrane, for example with cross-linking reagents^{1,2}, suggest that some membrane proteins occur in oligomeric form but more compelling evidence has come from studies of the solubilized membrane proteins. Only in the past decade, by more judicious handling of nonionic detergents, has the isolation of membrane proteins reached a stage at which the purified proteins can confidently be considered undenatured and intact. This has allowed the oligomeric states of some solubilized membrane proteins to be demonstrated by direct measurements of molecular weight. Based on data from such studies I suggest that oligomericity can be used as a criterion to determine whether a membrane protein isolated in these detergents is intact, because cleavage of oligomers by detergents often leads to denaturation.

A survey of some selected membrane proteins with oligomeric structure is given in Table 1. Although much evidence has been derived from chemical cross-linking procedures, these are not free from artefacts. Less equivocal data can be obtained with the isolated protein, by determination of the molecular weight (by ultracentrifugation) and of the number of active sites per protein. Any complication due to artificial aggregation of hydrophobic proteins can be largely excluded by the appropriate use of nonionic detergents. If the functional molecular weight calculated from the number of sites per protein agrees with that determined by hydrodynamic methods, there can be little doubt that the isolated protein is in the native oligomeric state.

In the intact membrane, cross-linking experiments for detecting oligomeric proteins have rarely been successful³. Nevertheless, erythrocyte band 3 protein (identified with the anion carrier⁴) has been shown to occur as a dimer⁵ and to span the membrane⁶. Also, $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ has been cross-linked⁷ in the membrane to form a dimer of the large α and β subunits, which indicates that this pump may exist as a dimer $(\alpha\beta)_2$. The ADP/ATP carrier in mitochondrial membranes can be shown by combined cross-linking and immunochemical methods to be a dimer (M. L. Vitali and H. P. Schultheiss, unpublished data). For Ca^{2+} -ATPase there is some evidence of an oligomeric state in the membrane but it is equivocal⁸.

More abundant and clear-cut evidence for oligomeric structure is provided by the isolated membrane proteins. If solubilized with nonionic detergents, these proteins form micelles with an increased particle size due to the bound detergent. The detergent obscures the true hydrodynamic properties of the

proteins but this can be allowed for by use of appropriate corrections⁹. The membrane protein preparations in the nonionic detergents are mostly monodisperse and, according to several criteria, are undenatured, retaining their native structure. Using Triton X-100 my colleagues and I have isolated seven different membrane proteins from mitochondria¹⁰⁻¹³, chloroplasts¹⁴ and bacteria^{15,16} and have determined their molecular weights by ultracentrifugation¹⁷⁻¹⁹.

The band 3 protein of erythrocytes isolated with Triton X-100 is a dimer—this agrees with *in situ* cross-linking studies⁵. However, if solubilized by acetic acid, a dynamic monomer-oligomer equilibrium is observed²⁰. This preparation may not be native according to the criteria described above. Among the mitochondrial proteins isolated with Triton X-100, the ADP/ATP carrier was first recognized as a dimer with two identical subunits^{17,21}. Extending these methods, other mitochondrial proteins such as cytochrome *b* (ref. 10) and the cytochrome *bc*₁ complex^{13,18} were purified in defined oligomeric states. The highly hydrophobic cytochrome *b* was characterized as a dimer¹⁰ and the cytochrome *bc*₁ complex, consisting of several different subunits, was found to be a dimer with each subunit present at least twice^{18,22}. Other dimers isolated in Triton include the cytochrome *c* oxidase complex²³, the uncoupling protein from brown fat mitochondria¹⁹, the acetylcholine receptor from postsynaptic membranes and the triose phosphate-*P*_i exchange carrier of chloroplasts¹⁴. Two membrane-bound dehydrogenases from bacteria, formate dehydrogenase¹⁵ and fumarate reductase¹⁶, which also form dimers, are not listed in Table 1 because they do not penetrate the cell membrane and bind only a relatively small amount of Triton.

When isolated with the nonionic detergent Lubrol, $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ is a dimer by ultracentrifugal studies^{24,25}; in the same detergent Ca^{2+} -ATPase is a tetramer⁸ but this may result from nonspecific aggregation as the tetramer can be dissociated into monomers by greater amounts of nonionic detergent

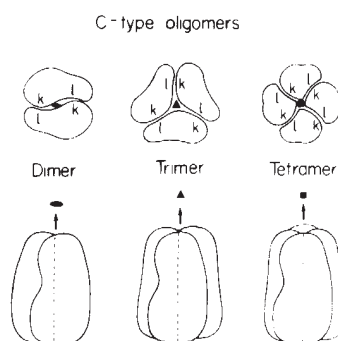


Fig. 1 Protein oligomers with *n*-fold rotational symmetry axis consisting of 2, 3 and 4 identical subunits. The repeating interfaces between the subunits are designated k and l.

Table 1 Some oligomeric transmembrane proteins

Protein	Source	Molecular weight ($\times 10^3$)		Type	Evidence (ref.)
		Monomer	Oligomer		
ADP/ATP carrier	Mitochondria: animal and <i>Neurospora crassa</i>	30 34	60 68	α_2 α_2	Inhibitor binding (21, 69) Cross-linking (17, 69) Hydrodynamic (17)
Uncoupling protein	Brown adipose tissue (mammalian)	32	64	α_2	Hydrodynamic (19)
($\text{Na}^+ + \text{K}^+$)ATPase	Plasma membrane, for example, shark rectal gland	—	330	$(\alpha\beta)_2$ $(\alpha\beta_2)_2$	Cross-linking (7) Hydrodynamic (24, 25)
Ca^{2+} -ATPase	Sarcoplasmic reticulum	100	400	α_4	
Anion carrier (band III)	Erythrocytes	90	180	α_2	Cross-linking (5)
Glucose carrier	Erythrocytes	55	100	α_2	Freeze-etching counts (33) Inhibitor binding (33, 70) Cited in ref. 71
Galactoside carrier	<i>E. coli</i>	30, 44?	—	α_2	
Porin	<i>E. coli</i> , outer membrane	37	—	α_3	Electron diffraction (72)
Bacteriorhodopsin	<i>Halobacterium</i>	28	—	(α_3)	Electron diffraction (28)
Acetylcholine receptor	Electric organ: <i>Torpedo</i>	250	500	$(\alpha\beta \dots)_2$	Hydrodynamic (73) Disulphide formation (74)
Cytochrome <i>b</i>	Mitochondria: animal and <i>N. Crassa</i>	30	60	α_2	Hydrodynamic (10)
Cytochrome <i>bc_1</i> -complex	Mitochondria	—	420	$(\alpha_2\beta \dots)_2$	Hydrodynamic (18, 22)
Cytochrome oxidase	Mitochondria	110	345	$(\alpha\beta \dots)_2$	Hydrodynamic (23)

without loss of ATPase activity²⁶. In contrast, rhodopsin occurs as a monomer both in the membranous and isolated states²⁷. The diffusibility of this protein as part of its function is in agreement with its monomeric state. Bacteriorhodopsin, which forms a crystalline purple membrane lattice²⁸, is also monomeric in detergent solution²⁹, although electron diffraction indicates trimer formation in the membrane.

Another fact which strongly supports dimeric structures comes from ligand binding studies, using highly specific inhibitors of translocators. The frequent result of such experiments is that only half a binding site exists per protein subunit: that is, a complete binding site requires at least a dimer of the protein subunits. Examples are the binding of ouabain to ($\text{Na}^+ + \text{K}^+$)ATPase³⁰ (see however ref. 31), the binding of atractylate or bongkrekate to ADP/ATP carrier^{21,32} and cytochalasin B binding to the glucose carrier³³.

There is evidence that the dimeric structure in the isolated protein is the native one for the ADP/ATP carrier as it loses ligand binding affinity on forming monomers. Only in the dimeric form does the cytochrome *bc_1* complex show succinate-cytochrome *c* reductase activity²². The ($\text{Na}^+ + \text{K}^+$)ATPase is reconstitutionally active when isolated with nonionic detergent as a dimer³⁴.

To conclude, note that some oligomeric membrane proteins have a rather cryptic transport function for ions: for example, cytochrome complexes may pump H^+ (ref. 35), various ATPases pump H^+ , Na^+ or Ca^{2+} , the acetylcholine receptor has a Na^+ channel³⁶, and the uncoupling protein of mitochondria catalyses anion transport³⁷.

Structural principles of oligomeric membrane proteins

Further analysis of the implications and characteristics of the oligomeric structure of membrane proteins requires a brief resumé of some general principles of oligomer formation. Much has been published on the symmetry properties of oligomeric enzymes and their functional consequences³⁸⁻⁴⁰, therefore these problems will be discussed only with regard to the particular situation of membrane proteins. Figure 1 shows some oligomers with 'C'-type symmetry. A closer look at the geometrical principle of association is important for understanding the characteristics of these structures. Dimeric and trimeric proteins can form only one type of symmetrical oligomer which is characterized by a C_2 - and C_3 -rotational axis. Tetramers can form several symmetrical structures; the oligomer with C_4 symmetry corresponds to those of the dimers and trimers. Higher symmetry of the D_2 type is found in many soluble tetrameric proteins. For reasons discussed below, this arrangement is

unlikely for tetrameric membrane proteins. Tetramers can also be visualized as being arranged in pseudo- C_4 symmetry and strict C_2 symmetry when two dimers associate to form a tetramer by retaining their original dimeric structure.

One point of discussion concerning oligomeric structure is that of isologous versus heterologous association and the relationship of this to cooperative effects between the subunits^{41,42}. Regarding this differentiation, only the dimer would be isologous whereas the C_3 -trimer and the C_4 -tetramer are heterologous. This differentiation, however, can be dismissed in analysing the contact area between the subunits in these oligomers. Each subunit may be thought to have a k- and an l-surface separated by the symmetry axis; in all three cases the k-surface associates with the l-surface of the other subunit in an 'heterologous' manner. This is true for the dimer in much the same way as for the trimer and tetramer. There is no particular distinction in the 'isologous' association of the dimer over those of the trimer and tetramer. In fact, one of the main advantages claimed for isologous as compared with heterologous association, which applies to all three cases, is that a mutation which changes the interface will be effective on both sides of the C-axis in the oligomer.

Oligomer membrane proteins and phospholipid bilayer

Membrane proteins are generally assumed to have a hydrophobic surface in those areas which make contact with the membrane lipid layer. The share of the hydrophobic surface should be particularly large in proteins deeply imbedded in the membrane, the most prominent example of which are carriers

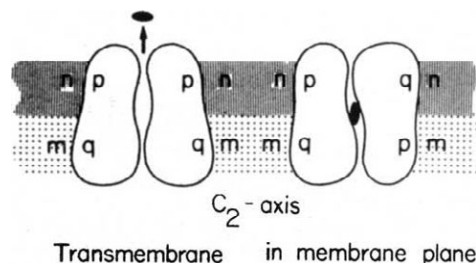


Fig. 2 Interaction of a dimeric protein with the asymmetric phospholipid bilayer. The localization of a dimer with the 2-fold axis either normal or parallel to the membrane plane. The surfaces of the protein are designated by p and q and the phospholipid bilayers by m and n.

such as those for ADP and ATP or glucose. The high amount of Triton binding to these proteins has been thought to reflect the extensive membrane contact area⁴³. However, the amount of detergent bound cannot be used as a measure of the hydrophobic region because of the tendency of nonionic detergents to form micelles at these surfaces⁴⁴.

The 'specific' interaction between subunits should be contrasted with nonspecific association often encountered in membrane proteins resulting from the interaction of the hydrophobic surfaces. This aggregation can occur if the isolated protein lacks sufficient detergent. The size of the aggregation may reach microscopic dimensions or it may be limited and even well defined, for example, in protein micelles with a hydrophobic core.

How are oligomeric membrane proteins arranged in the membrane? Biomembranes are topologically asymmetric for the obvious reason that they separate spaces of different composition. There is a prominent asymmetry between both leaflets of the lipid bilayer caused by asymmetric distribution of membrane phospholipid⁴⁵. The asymmetric assembly of the membrane can also include the membrane proteins. For the oligomeric transmembrane protein an arrangement with the rotational axis normal to the membrane is the obvious choice. This arrangement might be stabilized by interaction with the asymmetric bilayer, particularly by the different phospholipid head groups which can interact with the membrane protein through ionic forces.

Due to the interaction between phospholipids and protein, a transmembrane axial arrangement is energetically more favourable than the symmetrical arrangement with the axis within the membrane plane. This can be illustrated (Fig. 2) by summing up the free energy changes associated with the interaction of specific protein surfaces and phospholipid regions of the asymmetric bilayer. The free energy change for the transmembrane axial case (ΔG_T) can be described as being equivalent to $(np + mq) \times 2$ (see Fig. 2 legend for explanation of symbols) whereas the free energy change for the parallel axis would be $\Delta G_P = np + mq + nq + mp$. Assuming that $np + qm$ is the preferred interaction (a free energy minimum), for example, $np + qm > nq + mp$, it follows that $\Delta G_T > \Delta G_P$. These arguments are valid only for oligomeric proteins and we can conclude that the asymmetric transmembrane axial arrangement provides the membrane proteins not only with maximum interaction forces with phospholipids but also with additional stabilization of their quaternary structure.

After solubilization of membrane proteins in detergent, the asymmetric environment is abolished. By replacing the phospholipid bilayer, nonionic detergents form a micelle around the protein⁴⁴. In general the detergent will be asymmetrically distributed around the protein, but the uniform detergent does not allow a differential binding of molecules to different parts of the protein. The absence of this differentiation may contribute to the relative instability of detergent-solubilized proteins.

Membrane multicomponent complexes

A clear example of a C_2 -transmembrane arrangement of a dimeric membrane protein is the $(Na^+ + K^+)$ -pump (see also ref. 46). The sections responsible for ATP hydrolysis in both subunits must be directed towards the cytosolic face and electronmicroscopy shows that $(Na^+ + K^+)ATPase$ (and Ca^{2+} -ATPase⁴⁷) bulge only towards the inner side of the cell. Each large subunit of the $(Na^+ + K^+)ATPase$ binds a minimum of one 53,000 molecular weight (M_r) glycoprotein which faces the extracellular space^{31,48}.

In C_2 -transmembrane multicomponent proteins the subunits can be expected to be attached asymmetrically to the membrane, because identical intersubunit binding sites follow the transmembrane axis. Thus, in membrane-bound multi-enzyme complexes, subunits will be aligned in such a way that they form a C_2 -symmetric complex. Often only one or two of the peptides are imbedded in the membrane, the other subunits being attached to the surfaces protruding from the membrane,

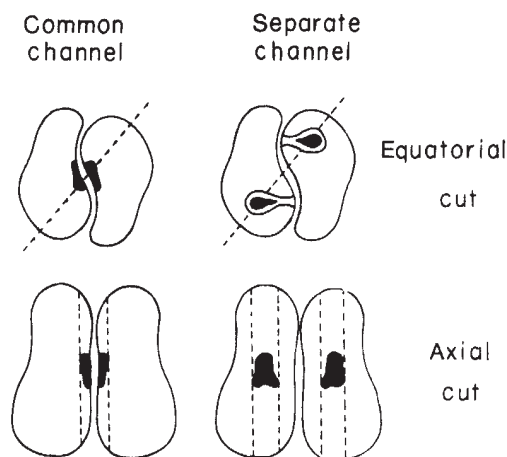


Fig. 3 Common central or separate channels and the binding centres in dimeric transport proteins.

for example in the ATP-synthase of mitochondria. Conversely, the existence of detachable subunits on one side of a membrane protein can be considered as evidence for C -type symmetry. For oligomeric membrane-bound glycoproteins, a transmembrane asymmetry is obligatory as the glycoside moiety is always directed towards the outer surface of the organelle membrane. This is the case, for example, with the anion channel of erythrocytes which is a dimer of two identical transmembrane glycoprotein subunits^{48,49}. Glycophorin may also form a transmembrane dimer in the erythrocyte, although no clear evidence has been reported⁵⁰.

Oligomeric structure and transport

So far we have seen that membrane proteins may acquire certain structural advantages unavailable to soluble proteins by forming oligomers in their hydrophobic environment. Do they also derive functional benefits from the oligomeric, axial transmembrane arrangement?

Translocation of hydrophobic molecules through the membrane requires a hydrophilic channel. Relatively rigid channels extending through the membrane exist in permanent forms to allow, for example, the nonspecific passage of solutes. Closely related to such pores are the gated channels through which, when open, many molecules or ions may diffuse. For the usual carrier-mediated transport, however, the channel should close or open during each turnover when one molecule or a defined set of ions is being moved through the membrane. In this case, the channels are highly flexible and gated so that at no instance do they freely extend across the whole membrane.

Binding forces between subunits are at a minimum along the symmetry axis because identical amino acid residues oppose each other. This should facilitate formation of a straight channel along the symmetry axis and corresponding pores have indeed been observed at atomic resolution for dimeric and tetrameric enzymes. With a transmembrane arrangement of the symmetry axis, a channel across the membrane could be formed in an oligomeric membrane protein (Fig. 3). Furthermore, this channel might have a hydrophilic surface as in membrane proteins a hydrophilic subunit interface may be energetically more favoured in the formation of oligomers, in view of the hydrophobic outer surface.

It has been tempting to suppose that membrane-bound carriers use the intersubunit channel as a translocation path for the substrate^{46,51} but experimental evidence in favour of such a model has only recently been obtained. The gated pore model was suggested from studies on the dimeric ADP/ATP carrier⁵², as will be discussed below. The alternative view has been that each subunit forms a separate channel. These channels could run either inside each subunit or at the interface (Fig. 3). However,

outside the symmetry axis, *a priori*, no straight path can be expected to occur.

Experimental data from exchange type systems⁵² suggest an intersubunit translocation path. (This is rather surprising because *a priori*, a reciprocating or 'flip-flop' mechanism of the two subunits would be the more obvious.) Two cooperating parallel channels are required for the simultaneous release and uptake of the exchange molecules. A dimeric carrier seems to be an obvious choice for this although alternatively the exchange could function in a sequential manner, often denoted as a 'ping-pong' mechanism. Each channel operates by facilitating, in turn, the uptake and release of a substrate molecule. The ping-pong performance is controlled by the gates in the channel. This exchange system differs from the unidirectional transport in that the gate can function only when the substrate is present. Either the common channel or the separate channels in a dimer could function by this process. Experimental evidence is largely in favour of this mechanism as opposed to the reciprocating model^{53,54}.

Binding sites and gates of the carrier

The translocation apparatus requires, besides the channel, a binding site for the substrate and a closely linked gate. It is the study of the binding sites that has so far produced the most valuable information concerning carrier mechanisms, for it is the binding sites of carriers which determine the high specificity of transport. It becomes abundantly clear from specificity studies that the binding of the transported molecule immediately triggers the transport reactions, presumably by means of conformation changes which open the channel gates. Substrate analogues or 'inhibitors' may bind with even higher affinity but have little or no triggering power.

In dimeric carriers, the two binding sites may be separated (Fig. 3), in which case the separate translocation paths would be imbedded within the subunits. With a central channel, the binding sites would directly face each other at the C_2 -axis and as a result, binding of one ligand should prevent that of the other. In fact, as shown above, in several dimeric carriers, binding of only one substrate or inhibitor per dimer has been observed.

The behaviour of some dimeric or tetrameric enzymes, in which negative cooperativity between the subunits controls binding and catalytic activity^{55,56}, has been called 'half-site' reactivity^{40,57}. Is the observed 'half-site' binding of carriers due to a similar effect? If so, little would be gained for dimeric carriers with separate channels, except for a decrease in transport activity. However, for the counter-exchange, 'half-site' reactivity would have a purpose if it generated a reciprocating

transport, which would be analogous to the flip-flop activity elaborated in detail for some half-site enzymes⁵⁷. The two binding sites are directed to opposite membrane sides and thus alternate between binding and releasing substrate to opposite sides. A flip-flop model has been discussed in detail for the $(Na^+ + K^+)ATPase$ ⁵⁸. Most of the evidence for $(Na^+ + K^+)ATPase$, however, is against counterflow and in favour of a sequential $(Na^+ + K^+)$ -exchange.

In the central channel model half-site reactivity becomes a trivial steric consequence as the two binding sites face each other. Binding of one substrate molecule would sterically prevent binding to the second site, particularly if the substrate is relatively large. As a result, functional asymmetry between both subunits is induced, which may further generate some conformational asymmetry.

The translocation process

The actual translocation process can be accommodated by assuming an alternating gating of the channel around the binding site such that the binding site is opened either to the internal surface of the membrane, when the carrier is said to be in the 'i' state, or to the external surface, when the carrier is in the 'e' state. In the case of an axial binding centre, the states are distinguished by a positional shift between the two subunits such that gates are closed or opened at the interface. With separate channels, a conformational change inside each subunit during the transition would be required and the gating can be thought of as being caused by positional shifts between the protein lobes around the channel or by discrete shifts of only one or two amino acid residues.

Evidence at the molecular level for the two translocational states of the carrier was first deduced by studying the binding site of the ADP/ATP carrier⁵². The transition between the 'e' and 'i' state (in that instance, 'c' = cytosolic state and 'm' = matrix state) had a strict requirement for substrate binding and as predicted, the binding centre was shown never to face both sides of the membrane simultaneously. These conclusions were fully corroborated with the solubilized, purified ADP/ATP carrier^{53,59}.

The radical change in specificity for the inhibitors of the binding centre on the transition between the 'c' and 'm' state has been interpreted, as illustrated in Fig. 5, to reflect the existence of a fixed rather than a rotational carrier⁵⁴. With the former an asymmetric substrate should, on entering the binding centre from one side, interact with the protein through a different face than on leaving from the opposite side. By contrast, with a rotational carrier the relative position of substrate to carrier should not be changed; in a stationary carrier the binding surface of the protein state should differ from that in the 'e' state so that there is a drastic difference in specificity. That is precisely what is seen with the ADP/ATP carrier which specifically binds atracylate in the 'c' state but bongkredate in the 'm' state, whereas the affinity for the substrates remains largely unchanged⁴⁷. Similarly, the glucose carrier in erythrocytes binds cytochalasin B only from inside⁶⁰ and binds various steroids asymmetrically from inside and out⁶¹; the Na^+ -dependent glucose carrier has a different ordered binding sequence for Na^+ and glucose on the sides⁶²; and the $(Na^+ + K^+)ATPase$ exhibits affinity changes for Na^+ and K^+ in the E_1 and E_2 state (although here the superimposition of an ATP-driven transition complicates the same principle).

The model of Fig. 5 requires that a single binding site changes its specificity between the 'i' and 'e' states of the carrier. Alternatively there are two different binding sites in series along the channel, either axial or intrasubunit, with the substrate migrating between the two sites (Fig. 4). The different specificity would then result from the dissimilar configuration of the 'i' and 'e' site located in dissimilar regions of protein. However, from a comparative viewpoint this seems improbable because enzymes generally contain only one binding centre per subunit, with two different centres being regulatory.

A configurational change in a binding site between the 'i' and 'e' state implies that a partially different set of amino acid

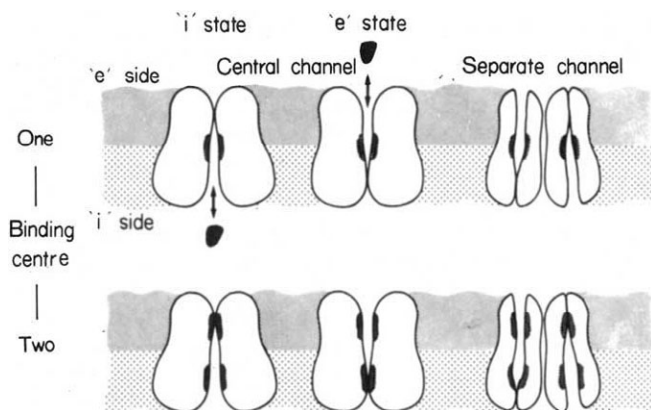


Fig. 4 Alternative 'gated pore' models of membrane carriers. With a single central channel the binding centre opens either to the outside (e-side) or inside (i-side) primarily by repositioning of the two subunits. For separate channels the gating requires intra-subunit conformational change.

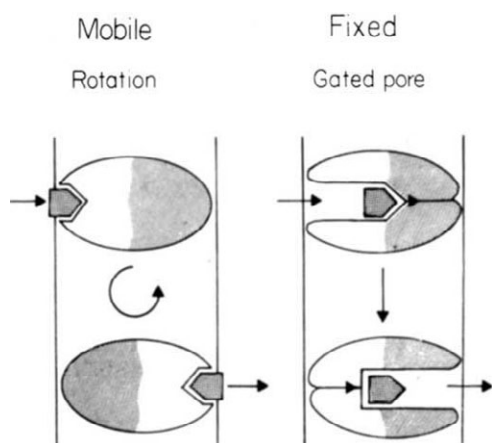


Fig. 5 The difference in flexibility of the binding centre in a rotational and fixed carrier⁵².

residues participates in the binding in the two states; the consequential change of affinity for the substrates could be beneficial in unidirectional transport. On the other hand, in counter-exchange, the binding affinities for the substrate would need to be well balanced, with energy-driven transport perhaps being achieved by a superimposed imbalance of the binding affinities in the two states. Evidence for the ADP/ATP carrier indicates that energy, as exerted by a membrane potential, does not change affinities but rather the distribution between the 'i' and 'e' states^{63,64}.

In trying to distinguish between the various models for translocation shown in Fig. 4, perhaps the best evidence for several dimeric carriers is that they bind only one molecule of substrate or inhibitor. Although this is still compatible with the existence of two separate channels in which the second site is inhibited by negative cooperativity, it seems unlikely because it has not been possible to increase binding at the ADP/ATP carrier by abolishing negative cooperativity, for example by monomerization of the carrier.

Another important observation is the conformational change on transition between the 'i' and 'e' state. This is most compatible with the central channel model where both subunits change conformation between the 'i' and 'e' state in parallel. More complicated assumptions must be made for the two-channel

model: conflicting cooperativity is required, a negative one to lock the other binding centre, and a positive one to synchronize the conformational transition between the binding and non-binding subunits. Furthermore, the reciprocating (flip-flop) model can be discounted because it requires that one subunit always exists in the 'i' state and the other in the 'e' state.

Most of these constraints are abolished if there are independent separate channels. In that case both binding sites of dimeric carriers should be active. Evidence for that exists for the anion carrier of erythrocytes⁶⁵ and the galactoside carrier of *Escherichia coli*⁶⁶ but has yet to be confirmed for the purified native protein.

Dimeric membrane proteins with other than transport functions, such as cytochrome complexes in mitochondria, also seem to show half-site reactivity. For example, cytochrome *b*, being a dimer in the cytochrome *bc*₁ complex, reacts as if there are two different species of cytochrome *b*. An anti-cooperativity between two identical cytochrome *b* molecules has been invoked for an alternating H⁺-pumping activity of cytochrome *b* in the redox cycle⁶⁷. A similar suggestion has been derived by Wikström⁶⁸ for the cytochrome oxidase complex. Note, however, that another H⁺-pump, bacteriorhodopsin, seems to operate with independent channels in each subunit. This can be reconciled with the fact that interaction between bacteriorhodopsin trimers in the hexagonal crystalline arrangement of the purple membrane is rather weak.

Conclusions

Oligomeric structures seem to be particularly well suited to integral membrane proteins which have carrier functions. The obvious role in transport of many integral proteins requires channels extending through the protein either along the *n*-fold axis or inside each subunit. The channels may be gated during each substrate turnover as in typical carrier-mediated transport. The catalytic binding centre in the channel of a number of dimeric carriers exhibits half-site reactivity which can be interpreted either as negative cooperativity for separate channels or as steric overlap in a common axial channel. Gating, as triggered by substrate binding, opens the site to the inside and outside, in turn. As a result the specificity of the carrier to inhibitor ligands is drastically changed in accordance with the direction in which transport is proceeding, for a stationary carrier. The parallel conformational change of both subunits between the 'e' and 'i' state, together with the half-site binding, can best be explained in terms of a single centre axial channel.

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Editor's Note: An article by Dr Jack Kyte of the University of California, San Diego, in which are presented some alternative views on the mechanistic role of membrane proteins in active transport, will be published shortly in *Nature*.

ARTICLES

The 'braided' F-ring of Saturn

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The larger of the two satellites which bound the F-ring of Saturn could have only one first-order resonance within the ring. If such a resonance exists, then a wave pattern of equally spaced loops which co-rotate with the satellite will be excited. This pattern could account for the braided appearance of the ring.

GOLDREICH AND TREMAINE¹ consider that particles in the narrow rings of Uranus are confined by tidal torques exerted on their orbits by small, unseen, satellites. The recent discovery by Voyager 1 of two small satellites, S13 and S14, which bound the narrow F-ring of Saturn², seems to confirm this view. However, the structure of the F-ring is complex and quite unlike that of the ϵ ring of Uranus. Some of the images of the F-ring show two bright rings which appear to be intertwined or braided. These two rings are closely associated with a third, but this is very diffuse (see Fig. 1)³. We show here how gravitational perturbation of the ring particle orbits by S13 and S14 may not only account for the confinement of the ring particles but may also explain some of the 'braided' structure of the F-ring.

Tidal torques

The tidal torque exerted on a narrow ring of particles of total mass m_r by a nearby satellite of mass m can be estimated from the scattering angle δ in Fig. 2b (ref. 4).

$$\delta = \frac{2Gm}{xU^2} \quad (1)$$

where the relative velocity U is given by

$$U = \pm \frac{3}{2}nx \quad (2)$$

G is the gravitational constant, n is the mean motion of the ring particles and x is the separation of the satellite and the ring. Since the tangential component of the relative velocity is reduced from U to $U \cos \delta$, angular momentum is exchanged with the net result that the ring experiences a torque

$$T = \pm \frac{4}{9\pi} \left(\frac{Gm}{nx^2} \right)^2 m_r \quad (3)$$

The sign of the torque depends on whether the satellite is on the inside or the outside of the ring and is such that the satellites repel the ring particles¹.

If a ring is bounded by two satellites, then its width will be reduced until the confining torques just counteract the tendency

of the ring to spread due to particle collisions. The equilibrium width of the ring is

$$W \sim \left(\frac{3\pi\tau}{4} \right)^{1/2} \left(\frac{x}{r} \right)^{5/2} \left(\frac{M}{m} \right) d \quad (4)$$

where M is the mass of the planet, r is the radius of the ring and d is the characteristic diameter of those particles which determine the spreading rate¹. τ is not simply the optical depth of the ring, this is always determined by the number density of the smaller particles, but the optical depth that the ring would have if all particles of diameter $< d$ were removed. For the F-ring of Saturn

$$d \sim \frac{40}{\tau^{1/2}} \left(\frac{30 \text{ km}}{W} \right) \left(\frac{m/M}{9 \times 10^{-9}} \right) \left(\frac{1,000 \text{ km}}{x} \right)^{5/2} \text{ m} \quad (5)$$

($m/M \sim 9 \times 10^{-9}$ corresponds to a satellite of diameter 200 km and density 1.2 g cm^{-3} .) If Goldreich and Tremaine are right, then the F-ring must have a substantial population of large particles. However, we consider that equation (3) may overestimate the magnitudes of the confining torques.

When the ring is in equilibrium, the torques due to the satellites must be equal and opposite, hence, from equation (3),

$$\frac{m_o}{x_o^2} = \frac{m_i}{x_i^2} \quad (6)$$

where subscripts o and i refer to the outer and inner satellite respectively. For the purposes of this article, it is sufficient to assume that the satellites and the ring particles move in coplanar, circular orbits.

Standing waves

Because $x \ll r$, the gravitational force on a ring particle due to a satellite is only effective at close encounters. During encounter, a particle initially moving in a circular orbit, acquires a radial velocity $U \sin \delta$ and thereafter moves in a keplerian ellipse of eccentricity

$$e \sim \frac{U \sin \delta}{nr} = \frac{4}{3} \frac{m}{M} \left(\frac{r}{x} \right)^2 \quad (7)$$

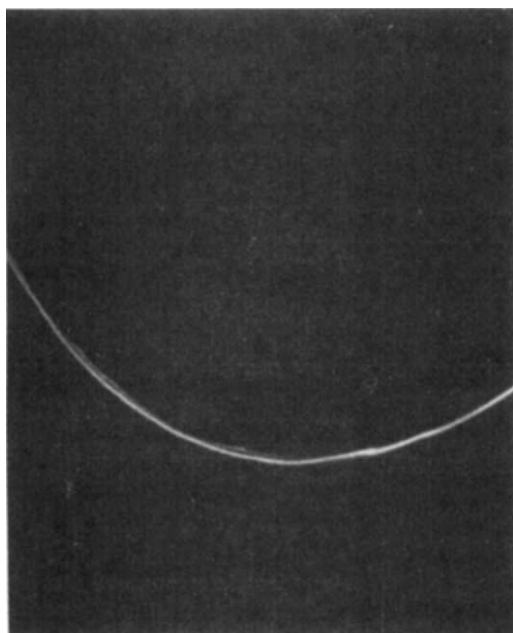


Fig. 1 This image, taken at a range of 750,000 km by Voyager 1, shows the highly complex structure of Saturn's F-ring. Two narrow 'braided' bright rings that trace distinct orbits are evident. Visible is a broader, very diffuse component, about 35 km wide. Also seen are 'knots' which are probably local clumps of ring material, but may be small satellites. Image courtesy JPL/NASA.

(see Fig. 2). A more accurate calculation by Julian and Toomre⁵ shows that the coefficient in this equation is 2.24 and not 4/3.

In a frame co-rotating with the perturbing satellite, all particles initially moving in circular orbits must follow identical paths after encounter. Thus each satellite generates a standing wave of amplitude

$$A = er \quad (8)$$

and wavelength

$$1 = \frac{2\pi U}{n} = 3\pi x \quad (9)$$

(see Fig. 2). In the inertial frame, each particle moves in an independent keplerian ellipse, but the pericentres of these elliptical orbits and the phases of the particles on the orbits are such that the locus of the particles is a sinusoidal wave which moves through the ring with the angular velocity of the perturbing satellite.

Because the tidal torques on the ring are equal, it follows from equations (6) and (8) that the amplitudes of the two waves are equal. For the F-ring of Saturn

$$A \sim 54 \left(\frac{m/M}{9 \times 10^{-9}} \right) \left(\frac{1,000 \text{ km}}{x} \right)^2 \text{ km} \quad (10)$$

and may be comparable with the width of the ring ($W \leq 30 \text{ km}$) (ref. 3). The interaction of these two waves, which probably have quite different wavelengths, could produce novel phenomena such as beats.

Resonance

Because $W \ll x$, the perturbations of the ring particle orbits will be highly coherent and although particle collisions will act to damp the waves, the process may be slow (and for this reason the magnitude of the torque given by equation (3) may be an overestimate). If the waves can survive from one encounter to the next, then we must consider the possibility of resonance.

This will occur if the ring circumference is an integral number of wavelengths, that is, if

$$\frac{2r}{3x} = p + 1 \quad (11)$$

where p is an integer. Consecutive perturbations will then be in phase and a wave of amplitude significantly greater than A may result.

The exact resonance condition can also be written

$$\frac{n'}{n} = \frac{p}{p+q} \quad \text{or} \quad \frac{p+q}{p} \quad (12)$$

where n' is the mean motion of the perturbing satellite and $q = 1$ for the type of resonance (first-order) that we are considering. As p can take any integral value ≥ 1 , a large number of resonances may occur. These are separated by a radial distance s , where

$$s = \frac{3}{2} \frac{x^2}{r} \quad (13)$$

For the satellites which bound the F-ring,

$$x_0 \sim 2,500 \text{ km} \quad (14)$$

$$x_i \sim 800 \text{ km} \quad (15)$$

(ref. 2). Hence

$$s_0 \sim 68 \left(\frac{x_0}{2,500 \text{ km}} \right)^2 \text{ km} \quad (16)$$

and

$$s_i \sim 7 \left(\frac{x_i}{800 \text{ km}} \right)^2 \text{ km} \quad (17)$$

These two sets of resonances will have quite different strengths. The amplitudes of the waves generated by a single encounter will be equal, but the larger satellite, which must be more distant

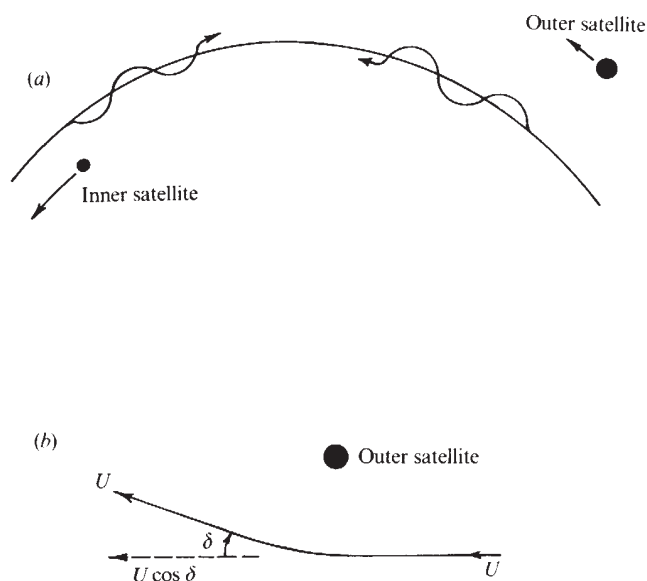


Fig. 2 Confinement of a narrow ring by two small satellites¹. *a*, Angular velocity decreases with increasing distance from the planet. Thus ring particles leading the outer satellite and lagging the inner satellite will be forced to move in eccentric orbits. Waves are excited which co-rotate with the perturbing satellite. *b*, The amplitude of the waves can be estimated from the scattering angle δ (ref. 2).

from the ring than the smaller satellite (see equation (6)), will have the larger relative velocity U and the time between encounters will be correspondingly shorter. Thus one would expect the resonances associated with the larger, outer satellite to dominate the structure of the ring. But these resonances are separated by a distance which is greater than the width of the ring. Thus, this satellite could have one, but only one, first-order resonance within the ring. The other possibility is that the width of the ring is < 7 km, and that a single first-order resonance associated with the smaller satellite exists within the ring.

For particles close to the exact resonance, the resonance condition is

$$pn - (p+1)n' + \dot{\omega} = 0 \quad (18)$$

where $\dot{\omega}$ is the pericentre of a ring particle orbit. If the particle is locked in resonance, and the forced eccentricity is small, then the pericentres of the ring particle orbits are not aligned, rather $\dot{\omega}$, ω and the mean longitude, or phase, are such that conjunctions of a particle and the satellite always occur at an apse of that particle's orbit⁶. The magnitude of the forced eccentricity, in the absence of damping, is given by⁶

$$e = \left| \frac{1/2(m/M)f(p)n'}{(p+1)n' - pn} \right| \quad (19)$$

Thus e increases markedly as the exact resonance is approached. At the exact resonance, the phase of the response changes by 180° . Similar behaviour is observed in any driven harmonic oscillator. For particles outside the exact resonance conjunction always occurs at apocentre, whereas for particles on the inside of the exact resonance conjunction always occurs at pericentre. Thus, the satellite excites a wave pattern of equally spaced loops which co-rotate with the perturbing satellite (see Fig. 3).

If p is large, then the inside width W_1 of a loop, that is, the maximum width of the gap generated in the ring, is given by⁷

$$W_1 = 2.96 \left(\frac{m}{M} \right)^{1/2} r \quad (20)$$

Thus, the effect of a given first-order resonance is determined solely by the mass of the perturbing satellite and is independent of both p and x . For the F-ring of Saturn,

$$W_1 = 39 \left(\frac{m/M}{9 \times 10^{-9}} \right)^{1/2} \text{ km} \quad (21)$$

The magnitude of this gap should be compared with the amplitude A of the short-period perturbations (see equation (10)). Two regimes can be clearly identified. If $A \gg 1/2 W_1$, then loop formation will be forever frustrated by the large perturbations suffered by the ring particles at each passage of the perturbing satellites. However, if $1/2 W_1 \gg A$, then loop formation is inevitable.

From equations (10) and (20), it follows that for $1/2 W_1 > A$, we must have

$$p < 56 \left(\frac{9 \times 10^{-9}}{m/M} \right)^{1/4} \quad (22)$$

or

$$x > 1,700 \left(\frac{m/M}{9 \times 10^{-9}} \right)^{1/4} \text{ km} \quad (23)$$

Thus, provided that the perturbing satellite is not so distant that first-order resonance is not possible, that is, provided that

$$\frac{n'}{n} > \frac{1}{2}, \quad (n' < n) \quad (24)$$

then the more distant the perturbing satellite the more likely is loop formation. However, if the unperturbed width of the ring is W , then the probability P that a first-order resonance will exist

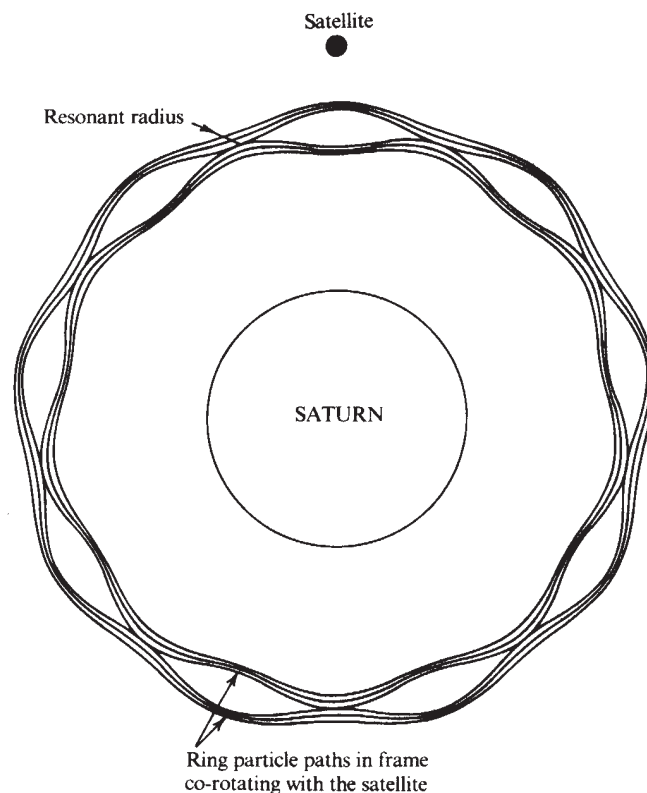


Fig. 3 Ring particle paths in a frame co-rotating with the perturbing satellite. Resonant gravitational interactions at radii in the ring where the ratio of the satellite and ring particle mean motions are close to $p/(p+1)$, where p is an integer, generate a system of waves which are stationary in the rotating frame. The waves on opposite sides of the exact resonance have a phase difference of 180° , hence the resultant wave pattern consists of $p+1$ equally spaced loops. This could account for the braided appearance of the F-ring of Saturn.

within the ring is given by

$$P = \frac{W}{s} = \frac{2rW}{3x^2} \quad (25)$$

and for the F-ring of Saturn,

$$P = 0.96 \left(\frac{W}{30 \text{ km}} \right) \left(\frac{1,700 \text{ km}}{x} \right)^2 \quad (26)$$

This suggests that loop formation is both possible and likely for satellites more distant than 1,700 km but not more distant than, say 5,000 km. According to early Voyager 1 reports², S13 orbits 2,500 km outside the F-ring, thus I consider that it is this satellite which is responsible for the 'braiding' of the F-ring.

Resonance is necessary to produce the bifurcation of particle orbits which is the essence of loop formation. The resonance need not be in the middle of the ring, in which case the two 'braids' will not necessarily be of equal brightness. Also, the short-period perturbations will not be negligible and, if the satellite orbits are not perfectly coplanar with the mean ring plane, waves both in the plane and out of the plane will be continuously excited by these satellites. The passage of these waves through the loops will give rise to shock phenomena associated with close-packing of ring particles⁷ and, possibly, because the ring is probably just outside Roche's limit for satellite formation, the formation of temporary particle clumps. Furthermore, because of these waves, the length and shape of

the loops will continuously change, and the structure of the ring, although periodic, will be highly complex and variable.

This theory cannot account for the third, diffuse ring. Perhaps this ring consists of small (micrometre-sized) particles which are generated in the main ring by erosion due to sputtering and meteoroid impacts and then removed by electromagnetic forces.

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Sequence and organization of the human mitochondrial genome

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The complete sequence of the 16,569-base pair human mitochondrial genome is presented. The genes for the 12S and 16S rRNAs, 22 tRNAs, cytochrome c oxidase subunits I, II and III, ATPase subunit 6, cytochrome b and eight other predicted protein coding genes have been located. The sequence shows extreme economy in that the genes have none or only a few noncoding bases between them, and in many cases the termination codons are not coded in the DNA but are created post-transcriptionally by polyadenylation of the mRNAs.

MITOCHONDRIA have a separate autonomously replicating DNA genome (for a review see ref. 1). Although most of the proteins present in the mitochondrion are encoded by nuclear DNA, a few are coded for by mitochondrial (mt) DNA and are synthesized by the separate mt translation system. The rRNAs and tRNAs of this system are mitochondrially encoded and it is likely that the other components, although nuclear coded, are distinct from those in the cytoplasmic translation system. The enzymes specific for the unique mitochondrial transcription and replication processes are probably also nuclear coded. We have recently shown that mammalian mitochondria have a different genetic code^{2,3} as have other mitochondria systems that have been studied⁴⁻⁷, and a different decoding mechanism⁷⁻⁹.

Replication of mammalian mtDNA proceeds by initiation of heavy (H) strand synthesis at a specific origin^{10,11} resulting in the formation of a displacement (D) loop with a newly synthesized H strand of ~680 bases known as 7S DNA. In mouse cells a large proportion of mtDNA exists in the D-loop form but only a few of these nascent H strands grow to full length. Initiation of light (L) strand synthesis is at a specific origin¹² and does not occur until this region has been exposed by H-strand synthesis.

Transcription of mammalian mtDNA is also unique in that both strands seem to be completely transcribed from promoters situated in the D-loop region^{13,14}. These primary transcripts are then processed to give the 12S and 16S rRNAs, tRNAs and a number of presumptive mRNAs, which are not capped but are polyadenylated¹⁵. The small size of the ribosomal RNAs, the chloramphenicol sensitivity of mitochondrial protein synthesis and the use of formyl-methionine in initiation of translation

have led to the suggestion that the mitochondrial genetic system arose by endosymbiosis of primitive bacteria within a host cell. The sequence of the rRNA genes, however, shows only low homology with present-day prokaryotic rRNAs¹⁶.

To understand this genetic system more fully we have determined the complete DNA sequence of both the human and the bovine mitochondrial genomes. We report here the sequence of human mtDNA and an interpretation of this sequence in terms of its coding capacity, gene arrangement and gene expression. The bovine sequence will be published elsewhere.

In the accompanying articles Ojala *et al.*¹⁷ and Montoya *et al.*¹⁸ report the sequence analysis of the stable polyadenylated RNAs. The comparison of the DNA and RNA sequences reveals the unique nature of the precise transcript processing in mammalian mitochondria.

Organization of the genome

The DNA sequence is shown in Fig. 1 along with the predicted coding sequences. This is presented schematically in Fig. 2. The identification of the genes and their precise location in the DNA sequence are based on several unique features, the evidence for which is discussed below and in the accompanying articles^{17,18}. The sequence shows extreme economy of organization in that there are none or very few noncoding bases between adjacent genes except for the D-loop region where we have been unable to assign any coding function. The sequence shown is that of the L strand which we define as the main coding strand containing the sense sequence of the rRNAs and most of the tRNAs and mRNAs. Thus, these RNAs are transcribed from (and therefore hybridize to) the H strand.

Origins of DNA replication

The 5' end of the major 7S DNA species found in the D-loop of HeLa cell mtDNA¹⁰ corresponds to nucleotide 191 of the DNA sequence. Other human 7S DNA species having different 5' ends have also been reported^{11,19,20}, but the precise location of these termini in the sequence presented here is not known. The size of the 7S DNA (≤ 680 nucleotides) and mapping data^{10,19,20}

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Table 1 Human mitochondrial genetic code

Phe	UUU	77	Ser	UCU	32	Tyr	UAU	46	Cys	UGU	5
	UUC	141		UCC	99		UAC	89		UGC	17
Leu	UUA	73		UCA	83	Ter	UAA	—	Trp	UGA	93
	UUG	16		UCG	7		UAG	—		UGG	11
Leu	CUU	65	Pro	CCU	41	His	CAU	18	Arg	CGU	7
	CUC	167		CCC	119		CAC	79		CGC	25
	CUA	276		CCA	52	Gln	CAA	81		CGA	29
	CUG	45		CCG	7		CAG	9		CGG	2
Ile	AUU	125	Thr	ACU	51	Asn	AAU	33	Ser	AGU	14
	AUC	196		ACC	155		AAC	131		AGC	39
Met	AUA	167		ACA	133	Lys	AAA	85	Ter	AGA	—
	AUG	40		ACG	10		AAG	10		AGG	—
Val	GUU	30	Ala	GCU	43	Asp	GAU	15	Gly	GGU	24
	GUC	49		GCC	124		GAC	51		GGC	88
	GUA	70		GCA	80	Glu	GAA	64		GGA	67
	GUG	18		GCG	8		GAG	24		GGG	34

The genetic code of human mtDNA differs from the universal code in that UGA codes for tryptophan and not termination, AUA codes for methionine not isoleucine, and AGA and AGG are termination rather than arginine codons. In addition, AUA and possibly AUU are initiation codons as well as AUG. Boxes of four codons are each read by a single tRNA with U in the first position of the anticodon; boxes of two codons are read by tRNAs with G:U wobble anticodons. The number of methionyl tRNAs and their codon response are unclear and discussed in the text. Also shown are the total number of each amino acid codon found in the genes and predicted genes shown in Figs 1 and 2. Codons ending in A and C predominate (36.37 and 40.93%, respectively) and those ending in U and G are used less frequently (16.33 and 6.36%, respectively).

indicate that the 7S DNA, and presumably the D-loop, do not extend into the tRNA^{Pro} gene. Although H-strand synthesis during replication initiates in the D-loop region, data from mouse cell mitochondria indicate that most of the 7S DNA molecules do not participate in DNA replication but rather are synthesized and then rapidly lost from the D-loop²¹.

The ~1,100-base pair region of the human mitochondrial genome between the tRNA^{Pro} and tRNA^{Phe} genes contains no large open reading frames in either strand. Furthermore, except for a small 7S RNA¹⁵, no large stable polyadenylated transcript has been found to map in this region. Unless RNA splicing is invoked it is extremely unlikely that this part of the genome codes for a major protein species. In the region covered by the D-loop, several blocks of nucleotide sequence homology exist between the human, bovine (S.A. *et al.*, unpublished results) and rat mitochondrial genomes²². However, the DNA separating these blocks is rather variable in length and exhibits no significant homology. The region between the 5' end of the 7S DNA and the tRNA^{Phe} gene seems to be one of the least conserved regions of the entire genome. This was initially detected by electron microscopy of heteroduplexes of sheep and goat mtDNAs²³ and has been confirmed by the DNA sequence comparisons. Very little sequence homology between the human, rat²² or bovine mitochondrial genomes can be detected in this region. The size of the region also seems to vary much

more than do intergenic spaces in other parts of the mtDNA, being 385 nucleotides in the human placenta, 291 nucleotides in rat liver and (based on alignment with the human and rat sequence) ~180 nucleotides in beef heart. This region may contain control signals for transcription of the mitochondrial genome.

L-strand synthesis during mtDNA replication initiates two-thirds of the way around the genome from the region of H-strand synthesis²⁴. In mouse cell mitochondria the DNA sequence immediately surrounding the L-strand origin is a simple inverted repeat that may form a hairpin loop structure when the L-strand origin is exposed by H-strand displacement synthesis during DNA replication¹². An analogous and highly homologous presumptive L-strand origin exists in the human mtDNA sequence (nucleotides 5,730–5,763) within a cluster of five tRNA genes. Interestingly, the differences between the human and mouse inverted repeats occur symmetrically, so that if secondary structure did exist, full base-pairing in the stem region would be maintained.

Ribosomal RNA genes

Eperon *et al.*¹⁶ were able to align the DNA sequence with the sequences of the 5' ends^{15,25} of the 12S and 16S rRNAs and to show that there were no noncoding bases between the tRNA^{Phe} gene and the 12S rRNA gene, and between the tRNA^{Val} gene

Fig. 1 The DNA sequence of human mitochondria (pages 459–461). The sequence of the L strand is numbered arbitrarily from the *Mbo*I-5/7 boundary⁵⁶ in the D-loop region. The sequence is displayed using the program TRANMT of Staden (unpublished) and shows the position of the genes for the tRNAs (boxed) and the rRNAs (underlined). The unidentified reading frames (URF 1–6) and identified protein coding genes are translated into the one-letter amino acid code (termination codons are denoted by an asterisk). The genes for cytochrome *c* oxidase subunits I–III are abbreviated COI–III and *cyt b* denotes the gene for cytochrome *b*. Which strand contains the sense sequence of the genes is denoted by → (L strand) and ← (H strand). The ends of the presumptive mRNAs transcribed from the H strand as determined by RNA sequence analysis^{17,18} are also shown below the sequence. The 5' end of the nascent H-strand (7S') DNA found in the D-loop is at nucleotide 191 (ref. 10). The origin of L-strand synthesis during DNA replication¹² is indicated by overlining of the possible base-pairing regions. The DNA sequence was derived mainly from a single human placenta mtDNA preparation⁵⁶ but some regions were determined on HeLa cell mtDNA. Restriction enzyme fragments of placental mtDNA were cloned in pBR322⁵⁶. Single-stranded template DNA was prepared by the exonuclease III procedure⁵⁷ and by cloning in bacteriophage M13 (refs 58–61). The DNA sequence was determined by the chain termination method of Sanger *et al.*^{62,63}. The sequence was analysed using the computer programs of Staden (refs 31, 55, 64 and unpublished). Nucleotides 10, 934–5, 14,272 and 14,365 were ambiguous and their identity assumed to be the same as in the bovine mtDNA sequence (S.A., M.H.L.B., A.R.C., I.C.E., F.S., I.G.Y., unpublished). The base composition of the L strand is 24.7% T, 31.2% C, 30.9% A and 13.1% G.

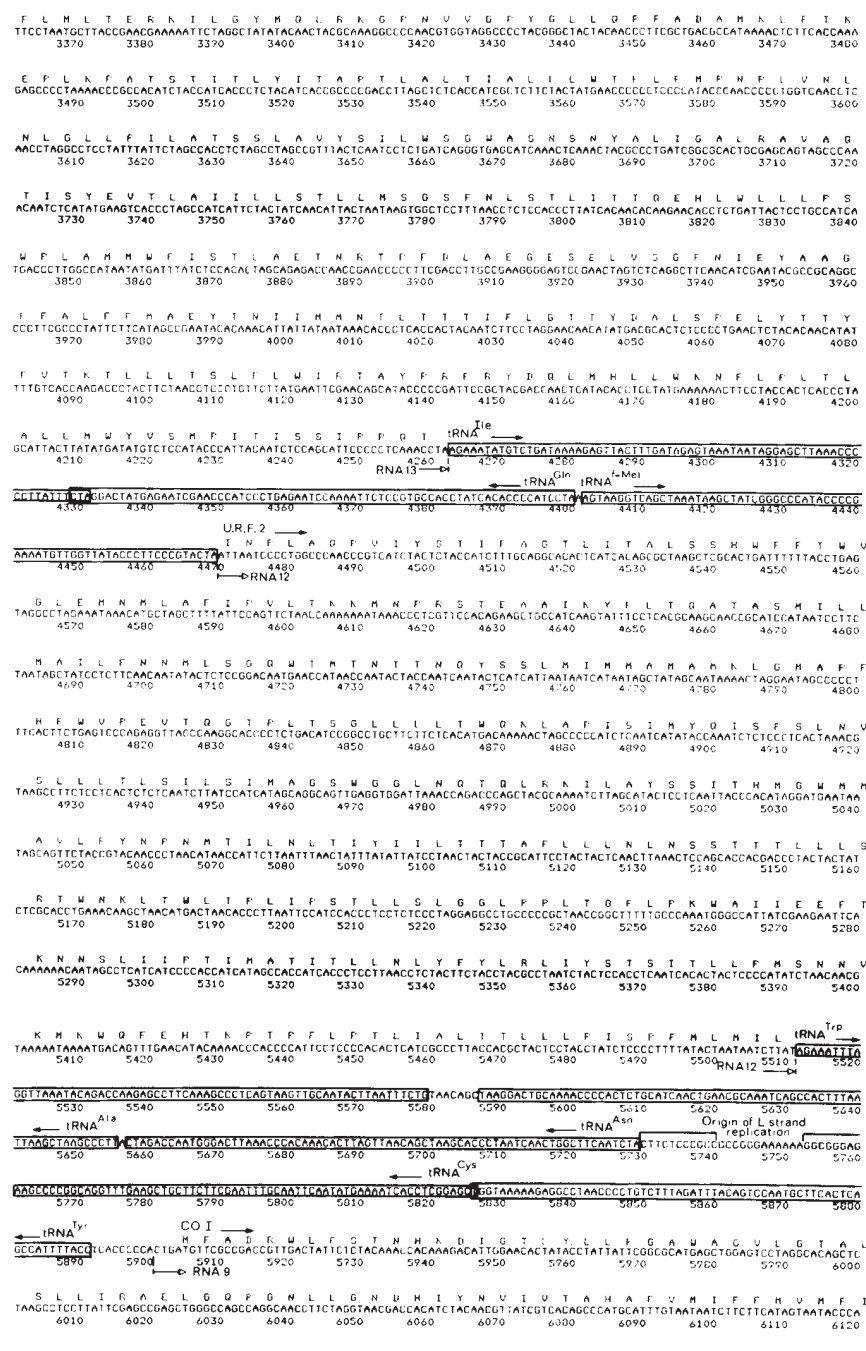


Figure 1

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Figure 1

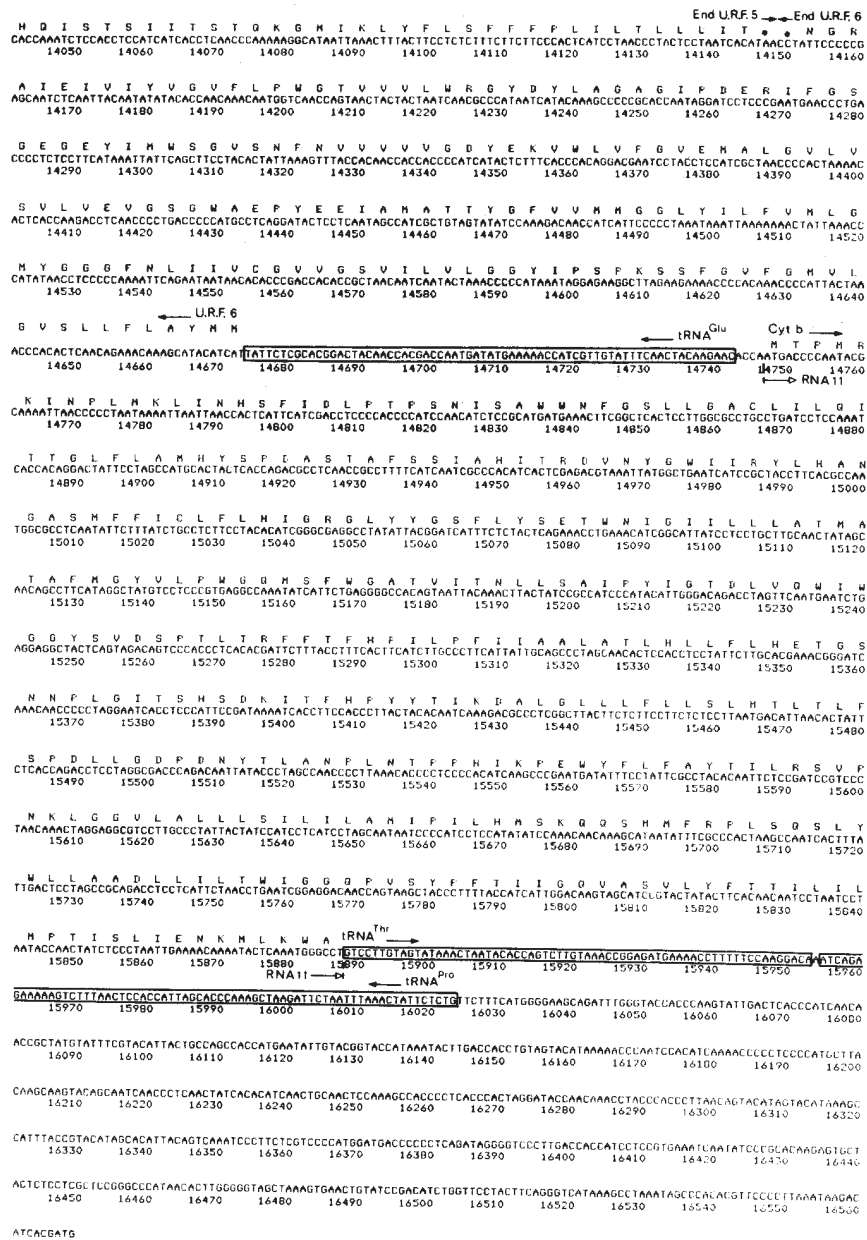


Figure 1

and the 16S rRNA gene. However, the 3' ends of the rRNA genes could only be predicted to be very close to the 5' ends of the flanking tRNA^{Val} and tRNA^{Leu} genes. It has since been shown that the mouse small subunit rRNA gene abuts the 3' tRNA^{Val} gene^{26,27} and alignment of a hamster large subunit rRNA sequence with the murine gene sequence suggests that there are also no noncoding bases between the large subunit rRNA gene and the 3' tRNA^{Leu} gene²⁸. Thus, the 12S rRNA and 16S rRNA and the flanking tRNAs in a primary transcript could be released by cleavage precisely at the 5' and 3' ends of the tRNAs without the need for further processing.

The high concentration of nascent transcripts from this region of the genome has led to the suggestion that there is a transcription attenuator in this region³⁰. This is supported by the observation that the hamster large subunit rRNA ends and is polyadenylated at any of four nucleotides within the last five nucleotides of the gene²⁸, which contrasts with the precise termini of the 12S rRNA and mRNAs^{29,30} which are presumed to be generated by processing of a primary transcript. It is possible that the 12S rRNA is also adenylated at the 3' terminus after transcription and processing; a single addition has been proposed for the hamster small subunit rRNA by comparison with the murine gene sequence³⁰.

The sequences of the rRNA genes were interpreted to provide support for a model in which mt ribosomes did not have sequence-dependent signals for recognition of the correct initiation codon but rather initiated at the first initiation codon in the mRNA¹⁶. This is consistent with the known 5'-end sequences of the mRNAs¹⁸ although not with the observation of single transcripts covering the URF A6L/ATPase 6 and URF 4L/URF 4 reading frames (Fig. 2) if the expected proteins are expressed.

The tRNA genes

Twenty-two tRNA genes have been identified in the human mtDNA sequence either by visual inspection or using the program TRNA³¹. Equivalent genes have been located in the bovine mtDNA sequence and most of these have been confirmed by direct sequencing of bovine mitochondrial tRNAs (B. A. Roe, E. Y. Chen, P. W. Armstrong and J. F. H. Wong, unpublished). We have been unable to substantiate a previous report⁸ which tentatively identified a gene for tRNA^{Met}. Apart from the tRNA^{Met}, these 22 tRNAs are sufficient to read all codons using a mechanism unique to mitochondrial systems⁷⁻⁹. No mammalian mt tRNAs have been shown to be imported from the cytoplasm (ref. 32 and B. A. Roe and E. Y. Chen, unpublished results). It had previously been assumed that the minimum number of tRNAs required to read all codons would be 32 for the 'universal' genetic code using G:U or I:A/C/U wobble³³. In human mitochondria the tRNAs for the two-codon families, that is, those genetic code boxes with two codons for one amino acid, have G:U wobble anticodons. However, only one tRNA is found for each of the four-codon families⁸ (see Table 1). These tRNAs have a U in the first position of the anticodon although each must read all four codons in its respective family. This is probably accomplished by U:N wobble⁸, although a two-out-of-three mechanism might operate³⁴. Although the modification pattern of the U in the first position of the anticodon of mammalian mt tRNAs is now known, it is likely that a similar situation exists to that found in *Neurospora* mitochondria⁷, whereby the tRNAs for the four-codon families have an unmodified U, but when the U is required for reading the two-codon families it is modified. This modification presumably prevents such tRNAs from reading codons ending in U or C.

Two species of mammalian mt methionyl tRNAs have been identified, a tRNA^{Met} and a tRNA^{Met} (refs 32, 35). Hybridization of these tRNAs to mtDNA showed partial additivity, indicating the existence of two separate genes³². In the DNA sequences we have only been able to identify one tRNA^{Met} gene, presumably coding for the initiator tRNA, based on its anticodon sequence CAT and the close homology of its anticodon arm with other initiator tRNAs (see ref. 36). The tRNA anti-

codon sequence CAU should normally be specific for AUG but as discussed below we believe that AUA and possibly AUU are also initiation codons in mammalian mitochondria. To recognize AUA (and AUU?) as well as AUG, the C in the first position of the anticodon would probably have to be modified. The AUA-specific tRNA^{Met} of *Escherichia coli* and of bacteriophage T4 have modified C residues in the first position of the anticodon^{37,38}, and hence represent examples of specific recognition of AUA by a tRNA with a CAU anticodon. Although potential tRNA^{Met}-like structures are found in the human and bovine sequences, none of these is particularly convincing nor are they conserved in the corresponding sequence. Also, extensive sequence analysis of the bovine mt tRNAs has not revealed any candidate for a tRNA^{Met} (B. A. Roe, E. Y. Chen, P. W. Armstrong and J. F. H. Wong, personal communication). If the gene exists in the mt genome it may have an unusual structure that is preventing its detection, for example, like that of tRNA^{Ser}_{AGU/C} which completely lacks the D arm³⁹. The other possibility is that one gene can give rise to both a tRNA^{Met} and a tRNA^{Met} by differential modification, although the hybridization data of Aujame and Freeman³² suggest otherwise. We are at present trying to clarify this problem by hybridization of labelled methionyl tRNA to restriction fragments of mtDNA.

The sequences of the mammalian mt tRNAs are unusual in that all, except tRNA^{Leu}_{UUR}, lack some or all of the following features found in other tRNAs: (1) the universal sequence G-T-ψ-C-R-A, (2) the constant seven-base length of the 'TψC' loop which in mammalian mt tRNAs varies between three and nine bases, and (3) the constant bases A₁₄, G₁₅ and G₁₈G₁₉ and the connections with U₈ and U₄₈ (yeast tRNA^{Phe} numbering system, see ref. 40). Thus, the mt tRNAs are apparently stabilized by fewer tertiary interactions. The most extreme case is that of tRNA^{Ser}_{AGU/C}, which lacks the D arm³⁹. Also, considering the similarity of homologous cytoplasmic tRNA species from different animals it is surprising that substantial variations are found when specific tRNAs from human and bovine mitochondria are compared. A compilation and comparison of these tRNAs will be published elsewhere. These missing or different tertiary interactions may mean that the mammalian mitochondrial tRNAs have more freedom than cytoplasmic tRNAs to evolve in certain areas, perhaps reflecting a different interaction with the ribosome. The role of the tRNAs in processing of the primary transcript is discussed below.

Table 2 Characteristics of human mt genes and comparison with bovine mt genes

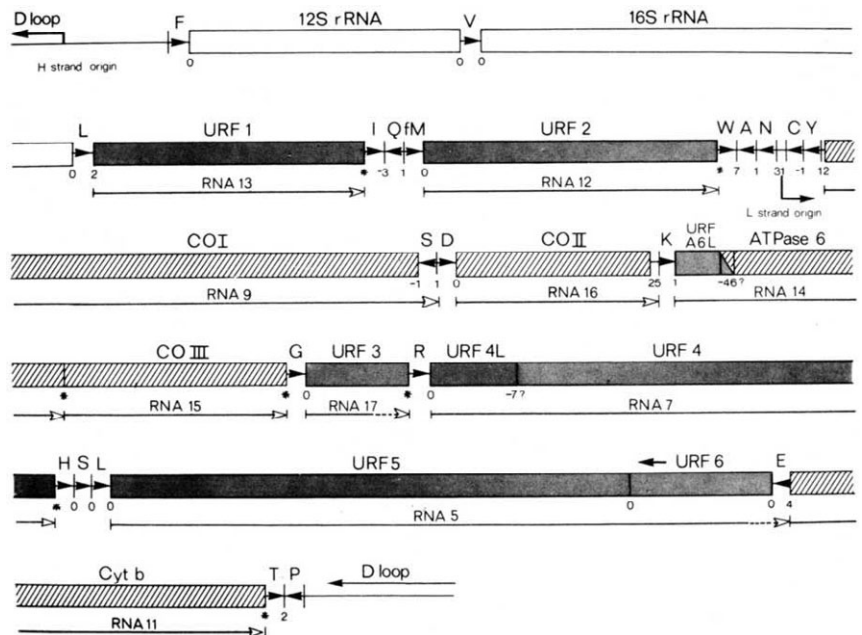
Gene	Protein length	Molecular weight	% Amino acid conservation	Initiation and termination codons	
				Human	Bovine
URF1	318	35,600	75.79	ATA *	ATG *
URF2	347	38,900	62.82	ATT *	ATA *
COI	513	57,000	91.23	ATG AGA	ATG TAA
COII	227	25,500	72.69	ATG TAG	ATG TAA
URF A6L	68	7,900	51.52	ATG TAG?	ATG TAA?
ATPase 6	226	24,800	77.88	ATG *	ATG *
COIII	261	30,000	86.97	ATG *	ATG *
URF3	115	13,200	73.91	ATA *	ATA *
URF 4L	98	10,700	73.47	ATG TAA?	ATG TAA?
URF4	459	51,400	74.07	ATG *	ATG *
URF5	603	66,600	69.49	ATA TAA	ATA TAA
URF6	174	18,600	62.64	ATG AGG	ATG TAA
Cytochrome b	380	42,700	78.10	ATG *	ATG AGA

The predicted length and molecular weight of the expected products of the human mt genes and unidentified reading frames were calculated from the DNA sequence using the computer programs of Staden (ref. 55 and unpublished results). Also shown is the percentage identical amino acids conserved between the human and bovine sequences calculated using the program AACHAN (R. Staden, unpublished). The predicted initiation and termination codons of the human and bovine genes are shown; * indicates that the termination codon is created post-transcriptionally by polyadenylation (see text).

Protein coding genes

Thirteen significant reading frames have been identified in the human mtDNA sequence, of which five have been assigned to

Fig. 2 Organization of the genome. A schematic representation of the sequence shown in Fig. 1. The tRNA genes are identified by the one-letter amino acid code and their sense sequence by either $\rightarrow$ (L strand) or $\leftarrow$ (H strand). Unidentified reading frames 1-5 (URF1-5) and the identified protein genes for cytochrome *c* oxidase subunits I-III (COI-III), ATPase 6 and cytochrome *b* (cyt *b*) all have their sense sequence on the L strand; URF6, however, is H-strand coded. The number of noncoding bases between genes is given below the junctions of the genes. A minus number denotes an overlap and an asterisk indicates that there are no noncoding bases at that point and that the termination codon for the preceding gene is created in the mRNA by polyadenylation (see text). The numbered RNAs are the presumptive mRNAs transcribed from the H strand which were aligned with the DNA sequence by hybridization to restriction fragments and by sequence analysis of their 5' and 3' ends (see accompanying articles^{17,18} for a more detailed transcription map showing the positions of the L-strand transcripts and the precursors to the H-strand transcripts).



known mitochondrially synthesized proteins. Only reading frames of at least 40 amino acids conserved with at least 50% amino acid homology in the bovine sequence were considered. The gene for cytochrome *c* oxidase subunit I (COI) was identified from an N-terminal amino acid sequence of the bovine protein (J. E. Walker, personal communication) and confirmed by comparison with the yeast mt COI gene. COII was identified from the complete amino acid sequence of the bovine protein⁴¹ and COIII by homology with the yeast gene⁴² and by an N-terminal sequence of the bovine protein (G. Buse, personal communication). The genes for ATPase 6 and cytochrome *b* were identified by homology with the corresponding yeast genes^{43,44} and also by amino acid sequences of bovine cytochrome *b* (W. Machleidt, personal communication). We have found no homology between the bovine amino acid sequence of ATPase 9 (E. Wachter, personal communication) and any of the reading frames, so that, as in *Neurospora*⁴⁵, this protein must be nuclear coded; in yeast it is mitochondrially coded^{46,47}.

In regions representing identified genes the respective human and bovine sequences are highly homologous. As shown in Table 2, there is ~80% amino acid conservation, which is 5% higher than the nucleotide conservation, reflecting the degeneracy of the genetic code. Over half the codons for the conserved amino acids are different in the third (degenerate) position or, in the case of the leucine codons UUR and CUN, in the first and third positions.

We have found eight unidentified reading frames (URFs) that are conserved in human and bovine mtDNA. On average, these show ~70% nucleotide and amino acid conservation (Table 2), again with approximately half of the conserved amino acids having changed codons. This type of homology is unlikely to have occurred by chance: for example, if the reading frames are compared out of phase in the +1 and +2 frames, the amino acid homology, including all the termination codons, is 35.4% and the percentage of conserved amino acids with changed codons drops to 4.5%. Therefore, we believe that most, if not all, of these reading frames represent genes for mammalian mitochondrial proteins. Further evidence in support of this is provided by the mapping and sequence analysis of the polyadenylated presumptive mRNAs reported in the accompanying articles^{17,18}. These RNAs correspond exactly to the reading frames of URF1, URF2, COI, COII, URF A6L and ATPase 6, COIII, URF3, URF 4L and URF4, URF5 and the antisense of URF6, and cytochrome *b* as shown in Figs 1 and 2. In addition, there are large L-strand transcripts covering the H-strand-coded URF6, and the number and sizes of these reading frames are in fairly good agreement with the band pattern obtained by

gel electrophoresis of HeLa cell mt proteins produced in the presence of emetine, an inhibitor of cytoplasmic protein synthesis (J. E. Walker, personal communication).

Two of the H-strand transcripts contain more than one reading frame, RNA14 (URF A6L and ATPase 6) and RNA7 (URF 4L and URF4). These present an anomaly and it is not known whether the reading frames are separated by a further processing event of these RNAs. The same organization is found in bovine mtDNA, making it unlikely that the breaks in the reading frames are due to sequence errors. A possible explanation is that these frames contain short intervening sequences which, when excised, leave only a single reading frame. However, using nuclease S₁ protein experiments, Ojala *et al.*⁴⁸ found no evidence for intervening sequences in the mtDNA regions coding for the polyadenylated RNAs.

Initiation codons

Although all the identified genes (except ATPase 6) and some of the URFs have an AUG initiation codon within the first six bases of their mRNA start, others do not. The first ATG in bovine URF2 is 320 codons downstream from the RNA start in a reading frame of 347 codons. Also, in human and bovine URF3 the first AUG is, respectively, 89 and 87 codons downstream in a reading frame of 115 codons. Ignoring the cases of ATPase 6 and URF4, which are the second reading frames in their presumptive mRNAs, and also the case of human URF2, which is discussed separately below, all reading frames that do not have an AUG near the 5' end of the RNAs have an AUA in an equivalent position. Thus, if these reading frames are translated completely, as their homology suggests they are, AUA must function as an initiation codon as well as AUG. AUA has already been shown to code for internal methionine as well as AUG^{2,3}. In the case of human URF2 there is an AUU in the same position as the AUA in bovine URF2. Thus, to translate completely this reading frame we must argue that, like AUA, AUU can also be an initiation codon in mitochondrial genes.

Termination of translation

Over half of the presumptive mRNAs do not have UAA or UAG termination codons at the end of the reading frame (Table 2). In three cases, human COI and URF6 and bovine cytochrome *b*, termination probably occurs at AGA and AGG codons. These have been predicted to be termination codons and not arginine codons as in the universal genetic code⁸. This is based on the observation that only CGN arginine codons are used in all the reading frames, and AGA and AGG are only found at the ends of reading frames at the junction with the next

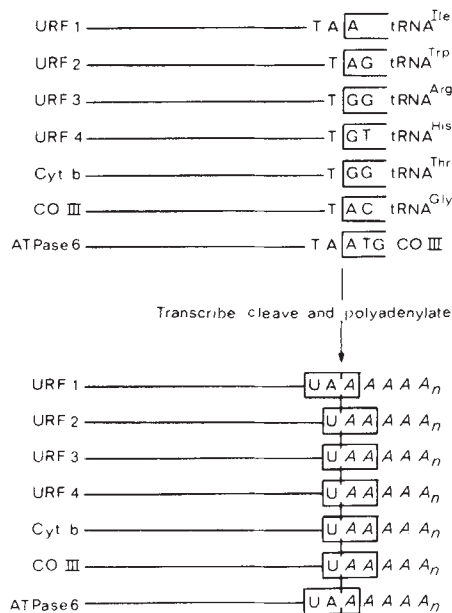


Fig. 3 The transcriptional processing and polyadenylation model for termination of translation.

gene or where the reading frame continues into the next gene. Also, no gene coding for a tRNA that could translate the codons AGA/G has been found. Finally, the composition of a C-terminal peptide fragment of bovine cytochrome *b* (W. Machleidt, personal communication) agrees with the prediction from the DNA sequence that this gene terminates in AGA.

To explain the lack of termination codons in most of the reading frames, we have proposed a model for the post-transcriptional creation of UAA termination codons in the mRNAs⁴⁹. This model was based on the observation that if the tRNAs were precisely cleaved out of a primary transcript by an RNase P type enzyme(s)^{50,51}, mRNAs from those reading frames with no termination codon would be left with either a U or UA at their 3' ends in phase with the reading frame. Thus, if these RNAs were then polyadenylated, a UAA termination codon would be created in-phase as shown in Fig. 3. Ojala *et al.*¹⁷, in the accompanying article, have confirmed this hypothesis by sequence analysis of the 3' ends of these mRNAs. This mechanism also provides the termination codon for ATPase 6. ATPase 6 and COIII have overlapping termination and initiation codons, that is UAAUG, and because the cleavage site is between the two A residues¹⁸ the termination codon is destroyed by RNA processing of the primary transcript (Figs 1, 2). However, as there is no tRNA gene in this region, the signal in the RNA sequence for processing is not known.

Transcriptional processing

As discussed above, comparisons of the human DNA sequence with the polyadenylated RNA sequences^{17,18} confirm the earlier predictions that the tRNA coding regions are involved in processing of the primary transcripts^{15,16,49,52,53}. It is now clear

that in the case of L-strand-coded tRNA genes the H-strand transcript is cleaved precisely at the 5' and 3' termini of the tRNA genes. Cleavage also seems to occur where the H-strand transcript has antisense, that is, H-strand-coded, tRNA genes. For example, cleavage of the H-strand transcript occurs between the antisense tRNA^{Glu} gene and the cytochrome *b* gene (see Figs 1, 2). In both cases the cleavage point is a few bases downstream from the antisense tRNA gene. An additional cleavage point is between the ATPase 6 and COIII genes, where there is no known tRNA structure. Thus, except for this latter case it is reasonable to assume that in the primary H-strand transcript L-strand-coded tRNA sequences can fold up and be recognized by an RNase P-like activity. Antisense (H-strand-coded) tRNA coding regions also seem to be recognized by the RNA processing enzymes, although cleavage points have only been found on the 3' side. With the exception the 12S rRNA and the tRNAs, the RNAs are then polyadenylated as discussed above; the tRNAs are matured by addition of CCA at their 3' terminus. At some stage base modification of the rRNAs and tRNAs must also occur. It is likely that the H-strand-coded genes in the L-strand primary transcript are similarly processed but this has been less well studied.

Mitochondrial origins

In conclusion, it is now clear that the mammalian mitochondrial genetic system cannot generally be classified as either prokaryote-like or eukaryote-like. The mammalian mt genetic code is different from the so-called universal genetic code and is read in a unique fashion by a minimal set of mitochondrial tRNAs. The mt tRNAs generally lack a number of features previously found to be invariant among tRNAs from all other sources, and the mammalian mt rRNAs have distinctive structures only distantly related to all previously determined rRNA sequences¹⁶. Similarly, the economy with which the large initial mitochondrial H-strand transcripts seem to be processed is unprecedented. It is also striking that mammalian mitochondria are very different from other mitochondria. In yeast mitochondria, for example, not only is there a slightly different genetic code, but also, the genes are widely spaced and in a different order, and in some cases they contain intervening sequences^{44,54}. These radical differences make it difficult to draw conclusions regarding mitochondrial evolution. Some form of endosymbiosis, involving the colonization of a primitive eukaryotic cell by a respiring bacteria-like organism, is an attractive hypothesis to explain the origins of mitochondria. However, the endosymbiont may have been no more closely related to current prokaryotes than to eukaryotes. It must also be borne in mind when making comparisons that the mechanism of selection and the selective pressures experienced by a captive cellular organelle may be quite different from those that affect a free-living organism.

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Distinctive features of the 5'-terminal sequences of the human mitochondrial mRNAs

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The 5'-end proximal sequences of all the putative mRNAs coded for by the heavy strand of HeLa cell mitochondrial DNA have been determined and aligned with the DNA sequence. All these mRNAs start directly at, or very near to, an AUG or AUA triplet, with the exception of one which starts at an AUU. The available evidence indicates that the terminal or subterminal AUGs and AUAs, and possibly also the terminal AUU, are initiator codons for the corresponding polypeptides. In most cases, the individual mRNA coding sequences are flanked on their 5' side by a tRNA gene, without any intervening nucleotide.

RECENT sequence analysis of human mitochondrial DNA (mtDNA) has revealed an extremely high degree of packing of genetic information in this DNA (ref. 1 and accompanying paper (ref. 26)). In agreement with these observations, a detailed transcription mapping study of HeLa cell mtDNA by the S_1 protection technique has shown that the sequences of the heavy (H) strand are almost completely saturated by the rRNAs, poly(A)-containing RNAs and tRNAs coded for by this strand^{2,3}. A particularly intriguing feature of the human mitochondrial gene organization is the frequent juxtaposition, or close proximity, of the individual protein-coding sequences, on their 5' side, with a tRNA gene or another reading frame. Thus, the gene for subunit II of cytochrome *c* oxidase (COII) has been shown to be immediately contiguous to a tRNA^{Asp} gene without any intervening nucleotide⁴. This unusual gene organization has raised the question of whether these putative mitochondrial mRNAs have, as all eukaryotic mRNA so far analysed^{5,6}, a 5' non-coding stretch, which may thus overlap the contiguous tRNA gene or reading frame, or whether they lack completely a leader sequence on their 5' side.

As a first approach to this question, the 5'-end proximal region of the putative COII mRNA has recently been sequenced and the sequence thus determined, aligned with the COII gene sequence⁷. The results clearly showed that the 5' end of the COII mRNA corresponds precisely with the first nucleotide of the COII coding sequence. These experiments left open the question of whether the complete lack of 5' non-coding nucleotides is a general feature of human mitochondrial mRNAs, reflecting stringent constraints in the initiation of mitochondrial protein synthesis or specific rules of RNA processing, or whether, on the contrary, the position of the initiator codon relative to the 5' end may vary in different mRNAs, possibly depending on the position of the adjacent tRNA gene or reading frame. We report here information bearing on these questions, obtained by sequencing a 5'-end proximal segment of all the H strand-coded, polysome-associated, poly(A)-containing RNAs, which are

presumably specific mRNAs, and by aligning the RNA sequences with the mtDNA sequence.

Isolation and 5'-end labelling of mitochondrial mRNAs

To isolate HeLa cell mitochondrial poly(A)-containing RNAs in adequate amounts for sequencing analysis, the micrococcal nuclease procedure for the purification of mitochondrial RNA⁸ was scaled up as previously described⁷. Figure 1a shows the electrophoretic pattern in a 1.4% agarose-CH₃HgOH slab gel, after ethidium bromide staining, of a large-scale preparation of mitochondrial oligo(dT)-bound RNA from micrococcal nuclease-treated organelles (from ~30 g of cells). One clearly recognizes the characteristic set of components previously

Table 1 5'-Terminal nucleotide analysis of *in vitro* labelled mitochondrial poly(A)-containing RNA species

Poly(A)-containing RNA species	% Of total ³² P radioactivity*			
	AMP	CMP	GMP	UMP
5	52.5	8.1	27.6	11.7
7	43.8	2.1	7.8	47.1
9	12.4	0.9	2.0	4.5
11	81.1	3.0	10.2	5.5
12	78.9	2.3	12.7	5.9
13	81.4	2.4	10.0	6.0
14	91.0	3.1	2.2	3.5
15	95.9	0.3	1.6	2.1
17	73.7	2.5	6.4	17.2

The 5'-terminal nucleotide was determined by exhaustive digestion of the *in vitro* labelled RNAs with nuclease P₁ (ref. 12, Calbiochem) and fractionation of the products on polyethyleneimine impregnated-cellulose TLC plates, as previously described⁸.

*The values refer to the radioactivity which migrated from the origin. The % of the input radioactivity recovered from the origin varied between 0.1 and 5%.

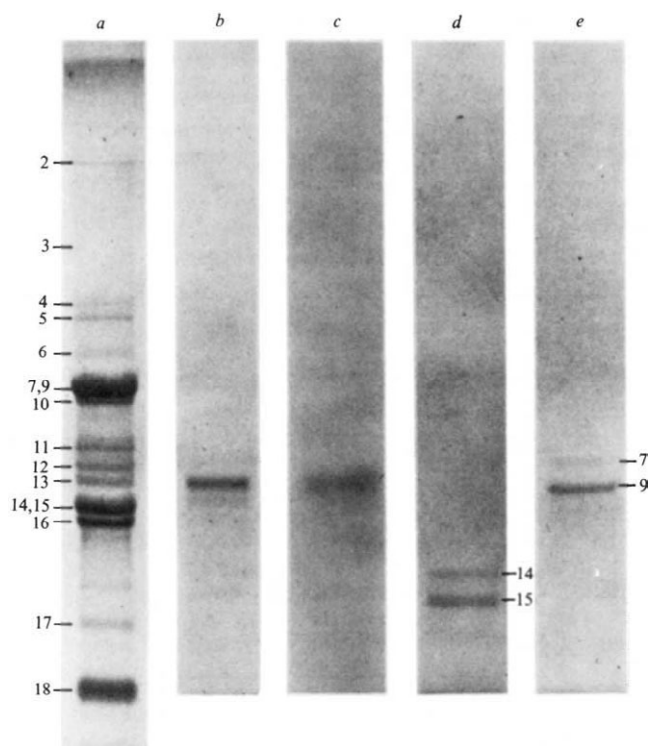


Fig. 1 Isolation and 5'-end labelling of mitochondrial poly(A)-containing RNA species. Lane *a*, electrophoretic pattern after ethidium bromide staining of the oligo(dT)-bound mitochondrial RNA fraction from 30 g of HeLa cells run through a 1.4% agarose-CH₃HgOH slab gel. Lanes *b* and *c*, RNA species 13 was eluted from several gel tracks similar to those shown in lane *a*, treated with RNase-free DNase and re-run on a gel in the same conditions (*b*), then eluted again, labelled at its 5' end with [γ -³²P]ATP and polynucleotide kinase after bacterial alkaline phosphatase treatment and re-run (*c*). Lanes *d* and *e*, re-run of RNA species 14 plus 15 (*d*) or 7 plus 9 (*e*) on 2% agarose-CH₃HgOH gels after elution from the gel track shown in lane *a* and treatment with DNase. Large-scale preparation of mitochondrial RNA by the micrococcal nuclease procedure^{7,8}, subsequent isolation and fractionation by 1.4% agarose-CH₃HgOH slab gel electrophoresis of the oligo(dT)-cellulose-bound RNA, elution, DNase treatment and re-run on agarose-CH₃HgOH slab gels of the individual components⁷, and 5' end labelling of the purified RNA species⁸ were as previously reported.

detected in ³²P-labelled preparations from HeLa cells⁹, with the exception of RNA species 1, which is extremely short-lived and present in mitochondria in very small amounts^{10,11}. Because of their relative abundance, their enrichment in partially purified polysomal structures⁹ and their relatively long half life^{10,11}, RNA species 5, 7, 9 and 11–17 in this set are probably specific mitochondrial mRNAs. Correlation of RNA sequence data, or of reading frames in mtDNA, with protein sequence data and with genetically characterized yeast gene sequences strongly supports the mRNA nature of several of these species^{1,2}. However, no direct *in vitro* demonstration of the messenger activity of these RNAs is available yet. Each of the putative mRNAs was eluted from the gel, treated with RNase-free DNase (to eliminate any contaminating DNA fragments deriving from the micrococcal nuclease treatment of the crude mitochondrial fraction) and re-run on a gel in the same conditions. As shown in Fig. 1*b* for RNA 13, a sharp band was observed at the expected position, and the RNA in this band was eluted. RNAs 7 and 9, and also RNAs 14 and 15, which were not separated from each other in the first electrophoretic run, could be resolved in a re-run on a 2% agarose-CH₃HgOH gel (Fig. 1*d*, *e*). Material purified as described above, from 40–250 g of cells, depending on the RNA species, was finally labelled at its 5' end with [γ -³²P]ATP and polynucleotide kinase after dephos-

phorylation with bacterial alkaline phosphatase, re-run on a CH₃HgOH-agarose gel (Fig. 1*c*) and eluted for the sequencing reactions.

RNA sequence analysis

The 5' termini of the isolated *in vitro* labelled RNA species were characterized first. As shown in Table 1, the distribution of radioactivity among the 5' mononucleotides indicated the adequate degree of purity of all the RNA species, with the exception of RNAs 5 and 7. RNA 5, which was the least abundant RNA species analysed, had slightly more than half of its radioactivity associated with pA, whereas RNA 7 seemed to consist of two components approximately equal in amount, one terminating in pA and the other in pU.

Sequencing of the 5' end proximal segments of the poly(A)-containing RNAs was performed by the methods of Donis-Keller *et al.*¹³ and Simoncsits *et al.*¹⁴, using cleavage of the RNAs by RNases T₁ (G-specific), A (C+U-specific) and U₂ (A-specific), followed by electrophoresis on polyacrylamide/urea gels⁸. The essential features of these RNA sequencing methods, which had been previously reported^{7,8,13,14}, were also observed here (Figs 2, 3, 4). RNases T₁ and U₂ were found to cleave regularly and exclusively after G and A, although with varying efficiency, probably depending on the surrounding sequence. RNase A cleaved exclusively after pyrimidines, but, as described previously, failed to cleave after some, especially when these were part of pyrimidine stretches. Accordingly, the absence of a band in any given position, after digestion with RNase A or U₂ or T₁, was tentatively interpreted as reflecting the presence at that position of a pyrimidine if the data indicated that this putative pyrimidine was part of a stretch of pyrimidines (two or more); in such a situation, the slowest moving among the pyrimidine-terminated oligonucleotides corresponding to the pyrimidine stretch was always clearly visible, with one or more of the other products being sometimes visible as fainter bands.

In most cases, a single band was observed at any given ladder position in the gel track, supporting the idea that a predominant RNA species was being analysed. In some cases, however, a definitely weaker band was observed at the same position as the predominant band, and disregarded as a contaminant (these weaker bands were often also present in the untreated RNA control). In a few cases, no band was seen in any of the lanes at a position corresponding to a ladder step, or two bands of comparable intensity were observed at the same position; in these cases, no conclusive identification was made.

Interpretation of the results was complicated by the presence of extra bands in the gel pattern, especially in the region corresponding to the smaller oligonucleotides. These extra bands were in general less strong than the *bona fide* specific RNase cleavage products. Because of this and because they did not correspond with bands visible in the ladder or corresponded with obviously abnormal steps of the ladder, these extra bands could usually be recognized as spurious. The nature of these extra bands is not clear. They may be due to the presence during the 5'-end-labelling reaction of contaminating RNA species or DNA fragments (deriving from DNA degradation during the DNase treatment step) in the RNA preparation, or they could reflect the formation, during the enzymatic or chemical cleavage of the RNA, of oligonucleotides with different phosphate end-groups (2', 3'-cyclic phosphate, 2' and 3' phosphate). The latter explanation seems the most likely to account for the fact that, in some sequencing analyses (RNAs 5, 7 and 17), both the specific RNase cleavage products and the chemical cleavage products gave doublets instead of single bands.

In each experiment, an untreated RNA sample was run in parallel with the RNase or chemically treated samples. Usually this control ('-Enz') did not show bands or exhibited them only in the region of the larger oligonucleotides.

The interpretation of the sequence of RNA 7 (Fig. 4) offered some difficulties because of the repetition of each band at the next position in the ladder. A similar observation had been made

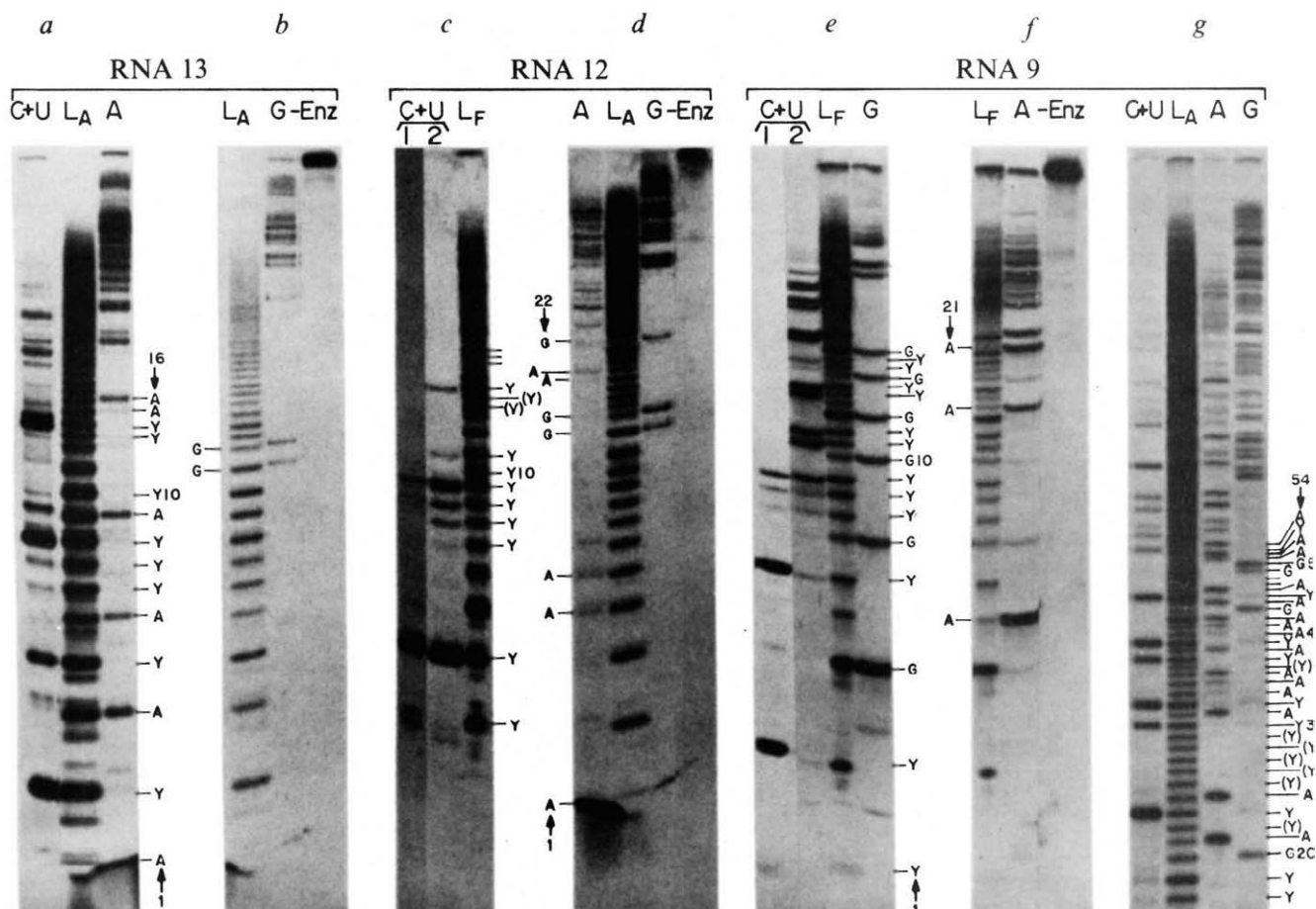


Fig. 2 Sequencing gels of mitochondrial poly(A)-containing RNA species 13, 12 and 9. *a, b*, RNA 13, 25% polyacrylamide/urea gel. Shown at the top of the gel are the cleavage specificities: RNase A (C+U), alkaline ladder (L_A), RNase U₂ (A), RNase T₁ (G) and RNA incubated without enzyme (-Enz). Y indicates a pyrimidine band. *c, d*, RNA 12, digested and analysed as indicated for RNA 13. The two left lanes in *c* show the digests obtained with decreasing amounts of RNase A. L_F indicates a formamide ladder. The horizontal ticks without a letter designation indicate ladder positions for which no nucleotide assignment has been made. *e-g*, RNA 9, digested as indicated for RNA 13 and analysed on a 25% (*e, f*) or, for the sequencing of residues from ~20 to 60, a 10% (*g*) polyacrylamide/urea gel. Details of the base-specific enzymatic cleavage reactions^{7,13,14} and the conditions of electrophoretic analysis^{7,8} are described elsewhere. In some experiments, to visualize a pyrimidine band in the first or second position of the sequence, the amount of RNase A used (per ~30 ng RNA and 3 µg yeast tRNA carrier) was increased to 20 ng.

previously for 12S rRNA⁸ and interpreted to reflect the existence of two species of 12S rRNA differing in molecular size by one nucleotide at the 5' end. With RNA 7, in agreement with the P₁ digestion data (Table 1), analysis of the sequencing gel showed that it could be interpreted as resulting from the electrophoretic separation of the products of RNase digestion of two species, one starting with AUG and the other with UG; comparison with the DNA sequence (see below) confirmed the validity of this interpretation. RNA 5 is present in HeLa cells in minute amounts, and only a partial purification could be obtained even after two electrophoretic runs through CH₃HgOH-agarose slab gels, as revealed by the P₁ digestion data (Table 1). Therefore, only partial sequence data could be obtained for this RNA species. However, we could identify with reasonable confidence the first two nucleotides (AY), the sixth (Y) and the ninth (G) (not shown).

Most mRNAs start at or very near an AUG or AUA triplet

Using the criteria described above, a stretch of 10–54 nucleotides, depending on the RNA species, could be read in the sequence of all the poly(A)-containing RNAs analysed here (except RNA 5). The sequences thus obtained are shown in Fig. 5; the corresponding DNA sequences (refs 4, 15 and 26 (accompanying paper)) are also reported to show their perfect alignment with the RNA sequences. Comparison of the RNA

and DNA sequences clearly indicates that all the AYG and AYA triplets underlined in the RNA sequences are indeed AUG and AUA codons. The partial sequence data for RNA 5 are consistent with the first triplet being an AUA. Thus, the striking results of this analysis are that, with one possible exception (RNA 12), all the putative human mitochondrial mRNAs start directly with an AUG or AUA triplet (which is a methionine codon in human mitochondria¹) or have a few nucleotides (1–3) preceding the first AUG or AUA. Sequencing data of human¹⁶ and bovine cytochrome *c* oxidase subunits I (COI) and II¹⁴ and comparison with the sequences of the yeast cytochrome *b* (A. Tzagoloff, personal communication) and cytochrome *c* oxidase subunit III (COIII)¹⁷ genes have indicated that the first AUGs of RNA 9 (COI mRNA), RNA 16 (COII mRNA), RNA 15 (COIII mRNA) and RNA 11 (cytochrome *b* mRNA) are initiator codons for the corresponding polypeptides. Thus, it seems reasonable to extrapolate from these results and interpret the 5'-proximalAUGs of the other mRNAs likewise as initiator codons. The mtDNA sequencing data suggest that AUA may function as an initiator codon in human mitochondria¹, and the data presented here support this. If the first AUA of RNA 13 is indeed the initiator codon, then, with the exception of RNA 12, all putative mitochondrial mRNAs have the initiator codon within the first six nucleotides. RNA 12 may also follow this rule. In fact, in bovine mtDNA, the first triplet of the reading frame corresponding to RNA 12 is AUA, instead of AUU as in human mtDNA; this observation sugges-

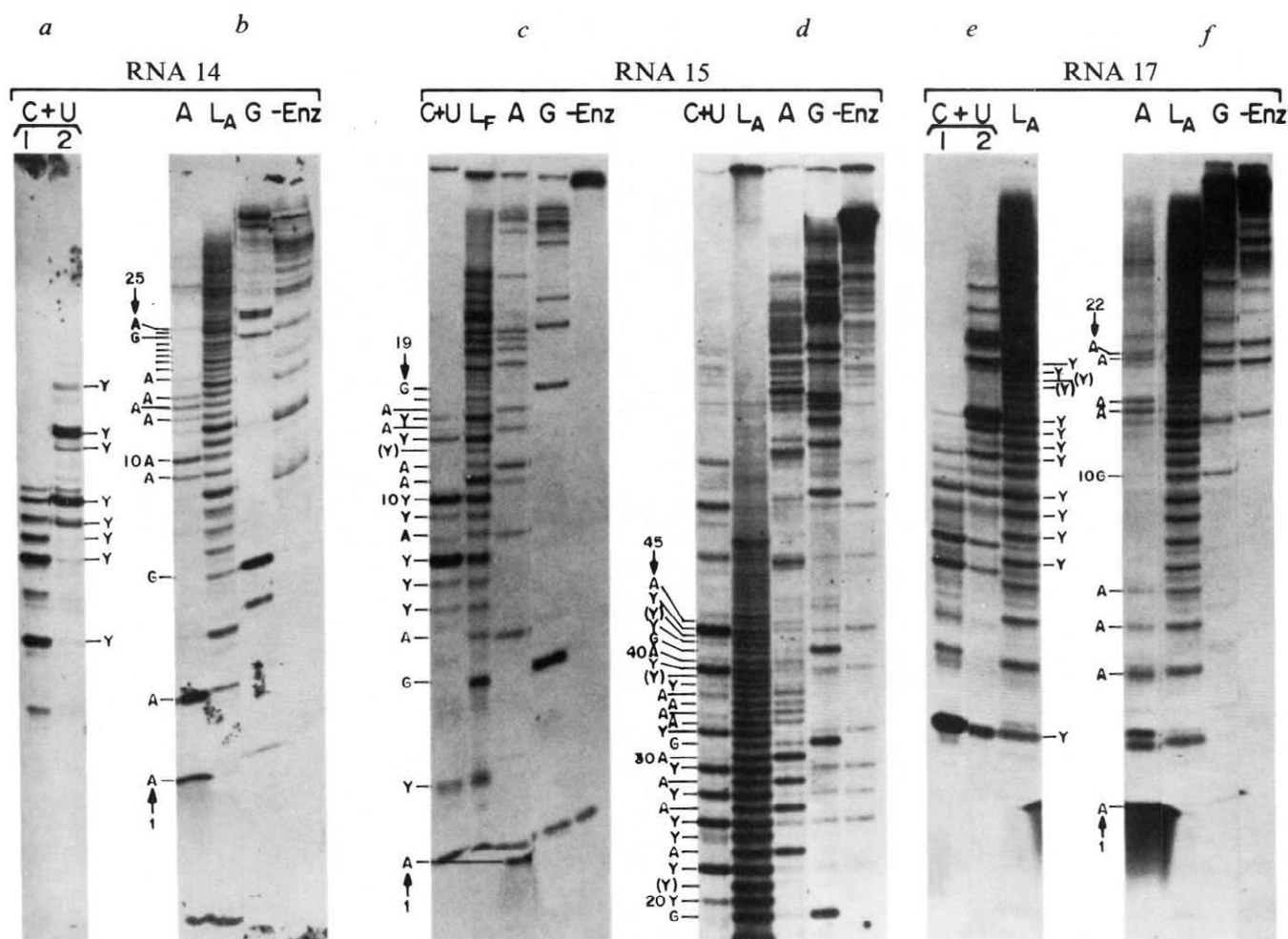


Fig. 3 Sequencing gels of mitochondrial poly(A)-containing RNA species 14, 15 and 17. *a, b*, RNA 14, 25% polyacrylamide/urea gel. *c, d*, RNA 15, digested as shown for RNA 14, and analysed on a 25% (*c*) or a 10% (*d*) polyacrylamide/urea gel. *e, f*, RNA 17, digested and analysed as described for RNA 14.

ted that AUU may function as initiator codon for the polypeptide coded for by RNA 12 (ref. 1). If the 5'-end heterogeneity observed in RNA 7 is an *in vivo* phenomenon, and not a consequence of degradation during the preparation and *in vitro* labelling of the RNA, and if the molecules starting with UG also function as mRNAs, the first initiator codon which could be used in these molecules would be the AUA occurring 14-nucleotides downstream from the 5' end.

How do ribosomes attach to mitochondrial mRNAs?

The finding that most mitochondrial mRNAs either start directly at the initiator codon or have only a few nucleotides (1–8) preceding this codon poses interesting questions concerning the mechanism whereby mitochondrial ribosomes attach themselves to these messengers. Both in eukaryotic and prokaryotic mRNAs, there is a stretch of variable length preceding the initiator codon^{5,6}. In prokaryotic mRNAs there is good evidence that this stretch contains a ribosome-binding site, which includes a properly positioned sequence complementary to the 3' end of 16S rRNA^{18,19}. A single exception to this rule is that of the λ repressor mRNA involved in repressor maintenance, which starts directly at the translation initiator codon²⁰. In this case, the low efficiency of translation of the mRNA was attributed to the lack of a strong ribosome-binding site. The observation that, in the 5' non-coding region of some eukaryotic mRNAs, there is considerable complementarity with a purine-

rich sequence near the 3' end of 18S rRNA⁵ has suggested that specific mRNA-rRNA base pairing may also help the positioning of eukaryotic ribosomes on the mRNAs; however, the variability in the position of the pyrimidine-rich sequence within the mRNA molecules and even the lack of these sequences in some mRNAs questions the generality of this mechanism⁶.

Sequencing analysis of a variety of eukaryotic (cellular and viral) mRNAs has shown that in most, if not all these mRNAs, translation starts at the AUG closest to the 5' end, apparently independently of the length of the 5' non-coding region (varying in the known cases between 11 and over 200 nucleotides) and of its nucleotide sequence^{6,21}. No minimum distance between the initiator codon and 5' end of the mRNA, which would allow proper initiation, has yet been defined for cellular or viral mRNAs; however, in the case of Sindbis virus RNA, there is evidence suggesting that the AUG immediately adjacent to the cap²² is not used for initiation⁶; this would imply that cytoplasmic ribosomes cannot recognize an initiator codon in such a location. To explain why initiation of translation is limited to the most 5'-proximal AUG, a 'scanning' mechanism has been proposed⁶, whereby a 40S ribosomal subunit would attach initially to the 5' end of an RNA chain and subsequently migrate towards the interior of the RNA, stopping when the first AUG is encountered; at this point the 60S subunit would join and translation start. In this model, the cap structure would enhance the binding of the 40S subunits to the 5' end of the mRNA, but would not be required. It is interesting that HeLa cell, and in

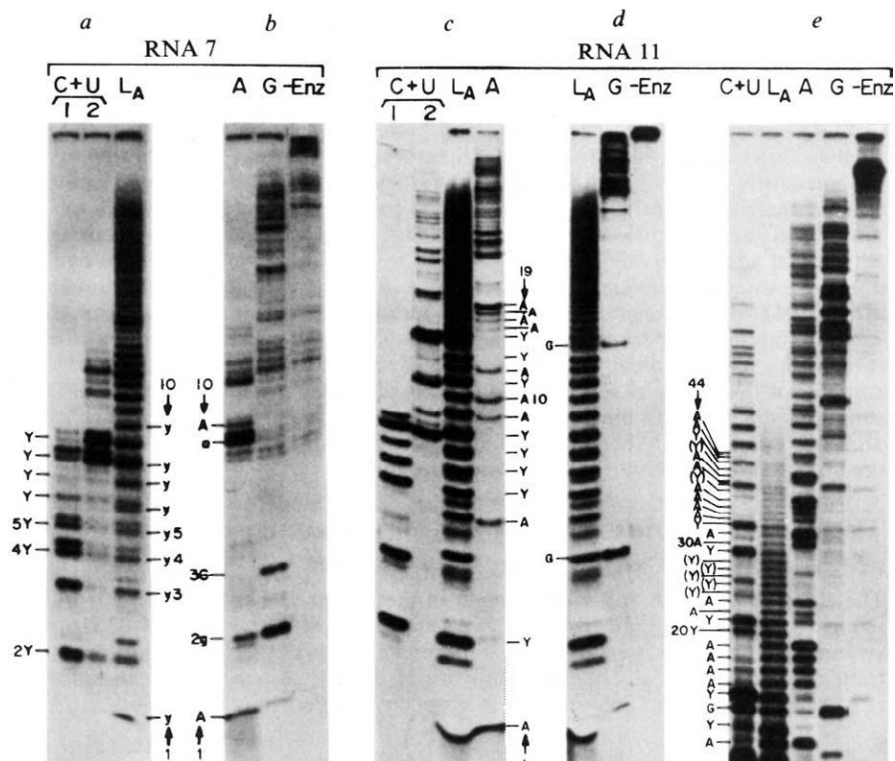


Fig. 4 Sequencing gels of mitochondrial poly(A)-containing RNA species 7 and 11. *a, b*, RNA 7, 25% polyacrylamide/urea gel. The capital and lower case letters indicate, respectively, the nucleotide assignments of the presumptively longer RNA species and of a species differing from this one by the lack of the 5'-terminal nucleotide at the time of labelling. *c-e*, RNA 11, digested as indicated for RNA 7, and analysed on a 25% (*c, d*) or a 10% (*e*) polyacrylamide/urea gel.

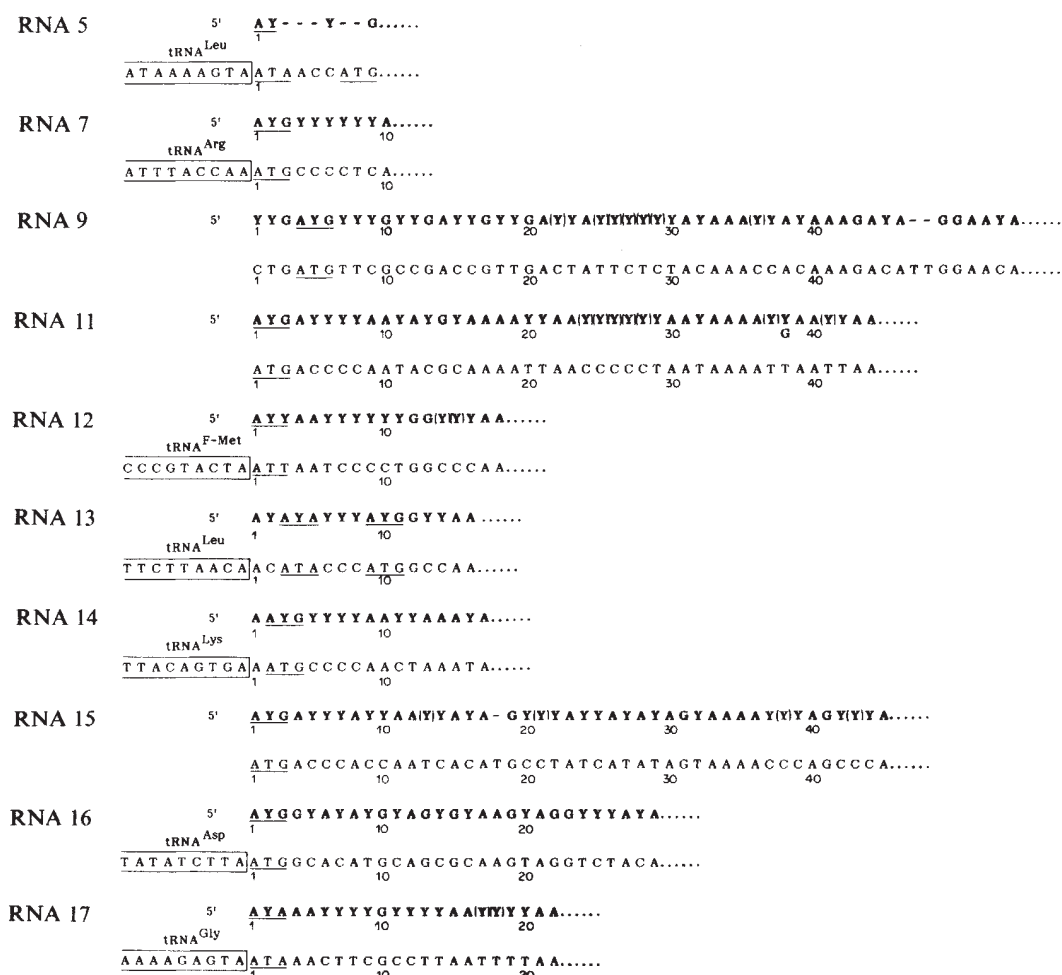


Fig. 5 The sequences of the 5'-end proximal regions of the mitochondrial poly(A)-containing RNAs determined in the present work are shown, aligned with the corresponding mtDNA sequences. A 3'-proximal segment of the sequence of the tRNA genes lying on the 5' side of most of the poly(A)-containing RNA coding stretches is also shown. Y indicates a pyrimidine (the parentheses denote a tentative identification, as explained in the text), a dash (—), an unidentified base; at position 38 in the RNA 11 sequence two possible assignments are indicated.

general, presumably mammalian, mitochondrial mRNAs are not capped²³.

The terminal or subterminal position of the initiator codon in human mitochondrial mRNAs may eliminate the need for a scanning process, at least an extensive one. A plausible mechanism would involve attachment of the ribosome at or near the 5' end of the mRNA with recognition of the initiator codon either directly or after a fine adjustment. The secondary structure of mitochondrial mRNAs may be such that it would exclude all internal sites containing AUG or AUA codons, exposing only the terminal or subterminal one. It is also conceivable that the special features of mammalian mitochondrial ribosomes or some initiation factor would make the ribosomes suitable for recognizing the terminal or subterminal initiator codons. Analysis of the potential secondary structures of human mitochondrial mRNAs and binding studies with mitochondrial or other ribosomes should elucidate the mechanisms operating in the initiation of translation in human mitochondria.

The 5' ends of most mRNA coding sequences are immediately adjacent to tRNA genes

The complete absence or minimal length of the 5' non-coding stretch in human mitochondrial mRNAs may represent the vestige of a primitive organization, or may reflect, with other traits (for example, the relatively small size of the rRNA and tRNA species), an evolutionary trend to simplicity and economy

of the mitochondrial genome²⁴. In any case, the degree of proximity of the initiator codon to the 5' end of the mitochondrial mRNAs seems to be dictated primarily by the features of gene organization of this genome, in particular, by the positions of the tRNA genes. In fact, whatever the location of the initiator codon relative to the 5' end of the mitochondrial mRNAs, the data presented here (Fig. 5) suggest that whenever a tRNA gene exists on the 5' side of the mRNA coding sequence, the latter starts immediately after the tRNA gene. This observation confirms and refines the results of a recent detailed transcription analysis of HeLa cell mtDNA³. These mapping and sequencing data strongly support the idea that the tRNA sequences represent the recognition signals for a putative processing enzyme which, by precise endonucleolytic cleavages, would release the 5' ends of the mature mRNAs^{2,3}. As will be discussed elsewhere²⁵, recent experimental evidence indicates that the tRNA sequences may perform a similar function in the processing steps which release the 3' ends of the human mitochondrial mRNAs. These striking features of the organization of the human mitochondrial genome underlie the crucial part that the tRNA sequences probably play in mitochondrial RNA processing in human cells^{2,3}.

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tRNA punctuation model of RNA processing in human mitochondria

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A 3'-end proximal segment of most of the putative mRNAs encoded in the heavy strand of HeLa cell mtDNA has been partially sequenced and aligned with the DNA sequence. In all cases, the 3'-end nucleotide of the individual mRNA coding sequences has been found to be immediately contiguous to a tRNA gene or another mRNA coding sequence. These and previous results indicate that the heavy (H) strand sequences coding for the rRNA, poly(A)-containing RNA and tRNA species form a continuum extending over almost the entire length of this strand. We propose that the H strand is transcribed into a single polycistronic RNA molecule, which is processed later into mature species by precise endonucleolytic cleavages which occur, in most cases, immediately before and after a tRNA sequence.

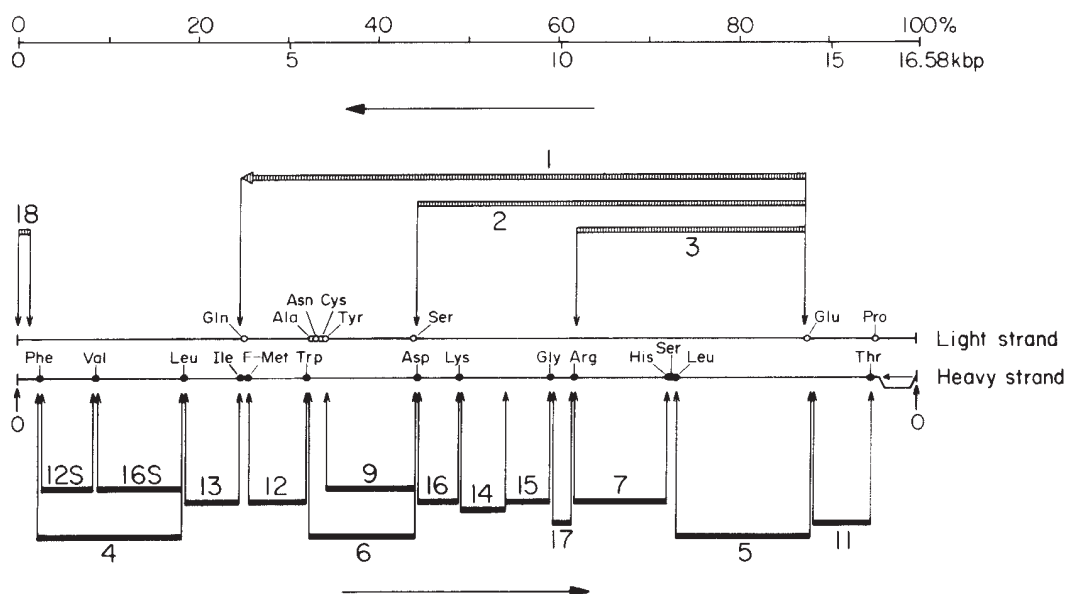
RECENT transcription mapping analysis of HeLa cell mitochondrial DNA (mtDNA) has revealed that the H-strand sequences specifying the rRNA and poly(A)-containing RNA species (which are probably specific mRNAs or their precursors) are flanked on one side, and often on both sides, by a tRNA gene^{1,2} (Fig. 1). In agreement with these observations, the alignment with the mtDNA sequence (refs 3–5 and 37) of a 5'-end proximal sequence of all H strand-coded putative mRNAs has shown that the sequences complementary to these mRNAs are almost always immediately adjacent, without any intervening nucleotide, to a tRNA gene or an mRNA coding sequence^{6,7}.

Here, we report that each of the eight mRNAs analysed is immediately contiguous, on its 3' side, to a tRNA gene or an mRNA coding sequence. On the basis of these and previous results^{2,4,6–8}, we propose a model of H-strand gene expression which involves transcription of almost the entire length of this strand in the form of a single polycistronic RNA molecule and its subsequent processing directed by the tRNA sequences.

Isolation and 3'-end sequencing analysis of mitochondrial mRNAs

The putative mRNAs were isolated from the poly(A)-containing RNA fraction of micrococcal nuclease-treated mitochondria

Fig. 1 Transcription map of HeLa cell mtDNA. The two mtDNA strands have been linearized at the origin of replication (0). The position and identities of the tRNA genes on the H strand (●) and on the L strand (○) were sequence (refs 16 and 37). The solid and hatched bars indicate the H-strand and L-strand transcripts, respectively; numbers refer to the various poly(A)-containing RNA species according to the classification of Amalric *et al.*¹⁹. The upper and lower arrows indicate the direction of L- and H-strand transcription, respectively. kbp, Kilobase pairs.



by electrophoresis through an agarose-CH₃HgOH slab gel, elution of the individual species from the gel, treatment with RNase-free DNase and re-run of each species on a separate gel of the same type, as described elsewhere⁶.

Partial sequence analysis of the mRNAs was carried out by an adaptation of the minus sequencing method using 'phased' oligo (dT) primers and reverse transcriptase^{9,10}, as described in Fig. 2 legend. In the transcriptase reaction, labelled primers and unlabelled dNTPs¹¹ were used rather than unlabelled primers and [α -³²P]dNTPs, as in the usual procedures; in the latter approach, the probable presence of contaminating oligonucleotides resulting from the DNase treatment (which could function as primers) was expected to complicate considerably the interpretation of the results.

To determine the correct primer to be used with the RNA being investigated (and thus the identity of the nucleotide adjacent to the poly(A) tail) each of three 5'-end-labelled primers [p(dT)₈-dA, p(dT)₈-dC and p(dT)₈-dG] was tested for its ability to prime cDNA synthesis using the RNA as a template. As shown previously^{9,10}, the presence of a specific nucleotide at the 3' end of the oligo(dT) stretch ensured that only the primer molecules hybridized with the RNA in phase (that is, those base-paired with the eight most 5'-proximal residues of the poly(A) tail and the first nucleotide on the 5' side of this tail) would promote extension synthesis by the reverse transcriptase. The band pattern formed by the products of this synthesis showed a highly variable intensity of the individual bands, and was very typical and highly reproducible for each RNA, thus providing a diagnostic test of a positive result; only limited extension synthesis was observed when the two other, non-specific primers were tested with the same RNA.

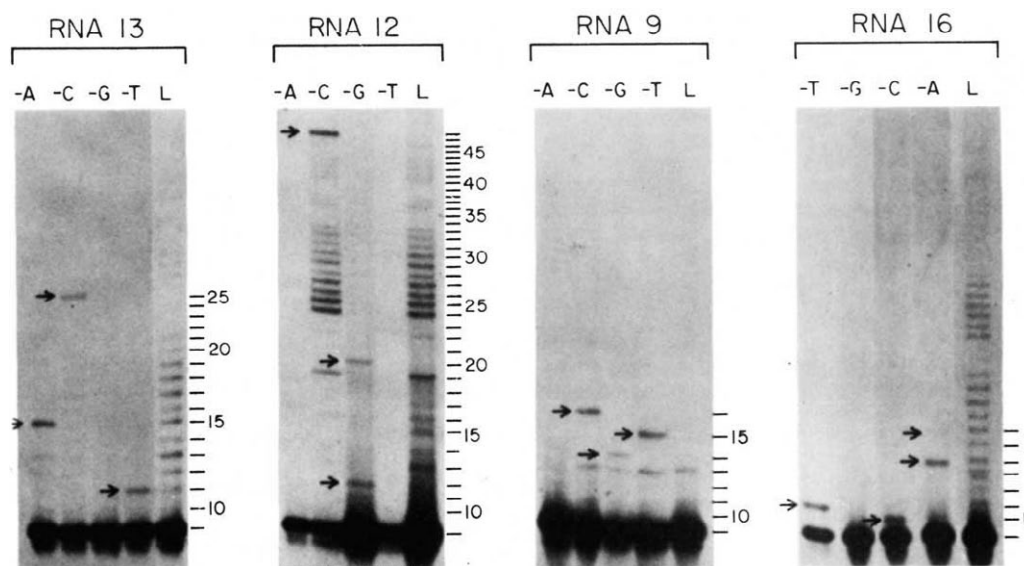
The correct oligo(dT)-dN primer identified for each RNA was incubated with the corresponding RNA, dNTPs and reverse transcriptase in four incubation mixtures, each lacking one of the four dNTPs. Figure 2 shows representative results obtained with some of the various putative mRNAs analysed. In general, in each 'minus' reaction mixture, a prominent band can be observed which corresponds to a specific ladder position. This band represents the oligo(dT) primer extended by the reverse transcriptase up to the position preceding, in the cDNA sequence, the nucleotide missing from the reaction mixture. For example, in the experiment using RNA 13, the '-A' lane shows a band at ladder position 15, the '-C' lane a band at position 25, and the '-T' lane a band at position 11. Therefore, the identities of the nucleotides at positions 16, 26 and 12 in the cDNA can be interpreted as A, C and T, respectively, and those of the

corresponding nucleotides in the RNA template U, G and A. The '-G' lane shows no band above the primer, which indicates the presence of a C residue at the position in the RNA sequence corresponding to step 10 of the ladder. The other RNA species gave results similar to those obtained for RNA 13. However, in some cases, an unambiguous identification of the stop positions corresponding to all four nucleotides could not be made. In particular, RNA 12 did not produce the expected band at position 10. Recent experiments suggest that this is probably due to the melting of the short extended primer (10 nucleotides long, comprising Ts and As) from the RNA template during the chromatography step. In the case of RNA 7, the strong signal from the band of the primer (position 9) prevented the recognition of the band at position 10. In these cases, a positive identification could not be made for position 10 or 11 in the cDNA. A faint second band was observed in some cases at a position higher up than that of the first band (see the '-G' lane for RNA 12 and the '-A' lane for RNA 16). This second band was tentatively interpreted to be due to partial readthrough by the transcriptase past the first stop position (presumably because of the presence of contaminating nucleotides in the reaction mixture) and arrest at the next stop position dictated by the sequence. In some experiments, multiple prominent bands were observed in an individual lane at positions below the stop band; these generally correlated with prominent ladder bands (for example, the '-C' lane for RNA 12), and were interpreted as reflecting pauses in the transcriptase progression, as previously observed¹², and were thus disregarded.

mRNA coding sequences are immediately adjacent at their 3' end to tRNA genes or other mRNA coding sequences

The partial sequencing data determined for the 3'-end proximal segments of the putative mRNAs are shown in Fig. 3. The corresponding DNA sequences (refs 3-5 and 37) are also reported to show their alignment with the nucleotides identified in the RNAs. Our experimental data allowed unambiguous matching of the RNA and DNA sequences. The only discrepancy observed is at a residue in RNA 11 corresponding to ladder position 13, where A was found instead of G. This may reflect a sequence difference between human placenta mtDNA (used for DNA sequencing) and HeLa mtDNA; such sequence differences have been previously observed at other positions⁴. It is interesting that the base substitution mentioned above does not change the codon assignment at that position (tryptophan). It is clear from

Fig. 2 Reverse transcriptase products in reactions using 'phased' oligo(dT) primers and poly(A)-containing RNAs 13, 12, 9 and 16. Partial sequence analysis of the 3'-end proximal segment of each RNA species was done by an adaptation of the 'minus' sequencing method using 'phased' oligo(dT) primers and reverse transcriptase^{9,10}, which will be described in detail elsewhere. Briefly, the p(dT)₈-dA, p(dT)₈-dC and p(dT)₈-dG primers were 5'-end labelled with [γ -³²P]ATP (8,000 Ci mmol⁻¹) and T4 polynucleotide kinase, after dephosphorylation with bacterial alkaline phosphatase⁸, then run on a



15% polyacrylamide slab gel in Tris-borate-EDTA. After autoradiography, the band corresponding to nonanucleotides in each lane, which contained most of the radioactivity, was eluted; the final specific activity of the labelled primers was $5-10 \times 10^7$ c.p.m. μg^{-1} . To determine the nucleotide adjacent to the poly(A) tail in the RNAs to be analysed, each RNA species (0.05–0.10 pmol) was mixed at 4 °C with a 100-fold molar excess of each of the 5'-end-labelled primers and 50 mM of each of the four dNTPs in 20 μl of 0.05 M Tris, pH 8.0 (25 °C), 0.05 M KCl, 0.005 M MgCl₂ and 0.01 M dithiothreitol. After addition of AMV reverse transcriptase (4 U), the reaction mixtures were incubated for 3 min at 39 °C. In some experiments, to facilitate the hybridization of the primer with the RNA, the mixtures were incubated for 5 min at 75 °C and then cooled for 15 min at 4 °C, before addition of the reverse transcriptase; however, no difference in results was observed. After stopping the reaction by cooling and addition of 0.01 M EDTA, the reaction products were precipitated with ethanol in the presence of 0.3 M NaCl and carrier yeast tRNA, the precipitates washed with ethanol, dissolved in 0.001 M Tris-EDTA, mixed with an equal volume of 10 M urea, heated for 2 min at 90 °C, then run on a thin (0.5 mm) 10% polyacrylamide/7 M urea sequencing gel for 3.5 h at 1,000 V. The correct oligo(dT) primer for each RNA, identified from the electrophoretic pattern of the products (see text: p(dT)₈-dA for RNAs 13 and 12, p(dT)₈-dG for RNAs 9 and 16), was incubated as described above with the corresponding RNA, dNTPs and reverse transcriptase in four reaction mixtures, each lacking one of the four dNTPs. In addition, a 'ladder' (L) reaction was performed in which all the four dNTPs were present. After stopping the reaction by quick cooling and addition of EDTA, excess primer was separated from the RNA-DNA hybrid by passage through a Sephadex G50 column equilibrated with 0.3 M NaCl, 0.01 M Tris, pH 7.4, in the presence of yeast tRNA carrier. The material in the void volume was ethanol-precipitated and then run on a 10% polyacrylamide/7 M urea sequencing gel. Arrows indicate the bands interpreted as corresponding to stop positions in the reverse transcriptase reaction. The numbering of the ladder steps indicates the molecular length (number of nucleotides) of the extended primers, starting from the 5'-end nucleotide.

Fig. 3 that the most 3'-proximal nucleotide identified directly in the RNA sequence either corresponds in the DNA to a residue immediately adjacent to the 5' end of a tRNA gene (RNAs 12, 16, 15, 7 and 11), or is separated from it by one to three As (RNAs 13 and 9). The most 3'-end proximal nucleotide identified in RNA 14 corresponds in the DNA to a residue separated by one A from the initiator codon (AUG) of the flanking RNA 15 coding sequence. Our experimental approach to the 3'-end sequence analysis did not allow discrimination between any 3'-terminal A encoded in the DNA and the As of the poly(A) tail. However, as in most of the mRNAs analysed, the most 3'-proximal nucleotide different from A corresponds to a nucleotide in the DNA immediately contiguous to a tRNA gene, it seems reasonable to extrapolate from these results and to assume that, in the other mRNA also, where one to three As separate this nucleotide from a tRNA gene or an mRNA coding sequence, these As are similarly transcribed from the DNA.

Comparison of the 3'-end proximal sequence of the small mitochondrial rRNA from hamster cells¹³ with the sequence of the corresponding segment of the human 12S rRNA gene⁴ shows a high degree of homology, with the most 3'-proximal nucleotide of the hamster RNA missing in the human mtDNA sequence which just precedes the tRNA^{Val} gene (Fig. 3). Therefore, it seems very likely that, as in mouse mtDNA¹⁴, the human 12S rRNA gene extends to the tRNA^{Val} gene, thus following the same rule described for the mRNA coding sequences. No direct information about the 3'-end proximal sequence of human 16S rRNA is available; however, comparison of the length of this RNA determined from S₁ protection data (~1,600 nucleotide)¹⁵ with the length of the interval between the tRNA^{Val} and the tRNA^{Leu} genes (1,559 nucleotides)⁴ strongly suggests that the 3'

end of the 16S rRNA corresponds to a residue in DNA immediately adjacent, or very close, to the tRNA^{Leu} gene.

In view of the exceedingly small amount of material available, no 3'-end sequence information was obtained for either RNA 5 or 17. However, the almost perfect correspondence between the molecular size of RNA 17, as estimated from S₁ protection data (340 nucleotides), and the length of the DNA sequence between tRNA^{Gly} and tRNA^{Arg} (346 nucleotides)², and the previously demonstrated juxtaposition of the tRNA^{Gly} gene with the 5' end of the RNA 17 coding sequence⁷ strongly suggest that the 3' end of the sequence specifying RNA 17 is immediately adjacent to the tRNA^{Arg} gene. Similarly, the mapping contiguity of the sequences coding for RNAs 5 and 11 (ref. 2) probably reflects their immediate juxtaposition at the nucleotide level.

Most mitochondrial mRNAs terminate with U or UA

Most of the 3'-end proximal sequences of the mitochondrial mRNAs terminate with U or UA (Fig. 3). The significance of this observation has been clarified by the analysis of the human mtDNA sequence (refs 16 and 37). It has been found that many reading frames, including those corresponding to the mRNAs mentioned above, lack a stop codon, and that, in these cases, a T or TA follows the last sense codon and immediately precedes a tRNA gene or another reading frame. This observation has led to the suggestion that poly(A) addition to the 3'-terminal U or UA of the transcripts of these reading frames may create the missing stop codon. The 3'-end proximal sequences determined here for the mitochondrial mRNAs strongly support this model for polypeptide chain termination in human mitochondria. The

reading frames corresponding to RNAs 9 and 16 have stop codons AGA and UAG, respectively^{3,5} (AGA is probably a stop codon in human mitochondria¹⁷). The observation that in these mRNAs there is a 3' noncoding stretch extending to the 5' end of the flanking tRNA gene indicates that, as in the case of the 5' terminus of the human mitochondrial mRNAs⁷, the rule dictating the possible presence and length of a 3'-end segment flanking the polypeptide coding sequence is the position of the tRNA genes in the DNA sequence.

The tRNA punctuation model of H-strand gene expression

The sequence data discussed above and in the accompanying paper⁷ and the results of previous work^{4,8} indicate that the H-strand sequences coding for the rRNAs, poly(A)-containing RNAs and tRNAs are immediately contiguous to each other, extending continuously from coordinate 2/100 to coordinate 95/100 (relative to the origin 0/100). This arrangement is consistent with a model of transcription of the H strand in the form of a single molecule which is processed by precise endonucleolytic cleavages before and after each tRNA

sequence to yield the mature products or, in some cases, processing intermediates, like the putative precursor of the rRNAs (RNA 4) and the precursor of RNA 9 (ref. 2).

Recent work has indicated a concentration of H-strand nascent transcripts in the quadrant of the mtDNA *Hpa*II map which is adjacent to the origin of replication in the direction of H-strand transcription¹⁸ (Fig. 4a). These results strongly suggest that the region of mtDNA around the origin of replication contains an initiation site for H-strand transcription—such a site may thus represent the promoter of the single transcript postulated here. Furthermore, the failure to detect any giant-size H-strand transcripts either associated with transcription complexes¹⁸ or as discrete species^{2,19}, in contrast to the occurrence of discrete giant-size L-strand transcripts, points to the possibility that the processing of the H-strand transcripts proceeds while they still reside on the mtDNA transcription complexes. These results taken together support a model in which transcription of the H strand starts near the origin of replication and proceeds uninterruptedly at least up to the distal end of the D-loop, while the growing chains are processed to yield the mature rRNAs, poly(A)-containing RNAs and tRNAs (Fig. 4b).

Hamster
small rRNA

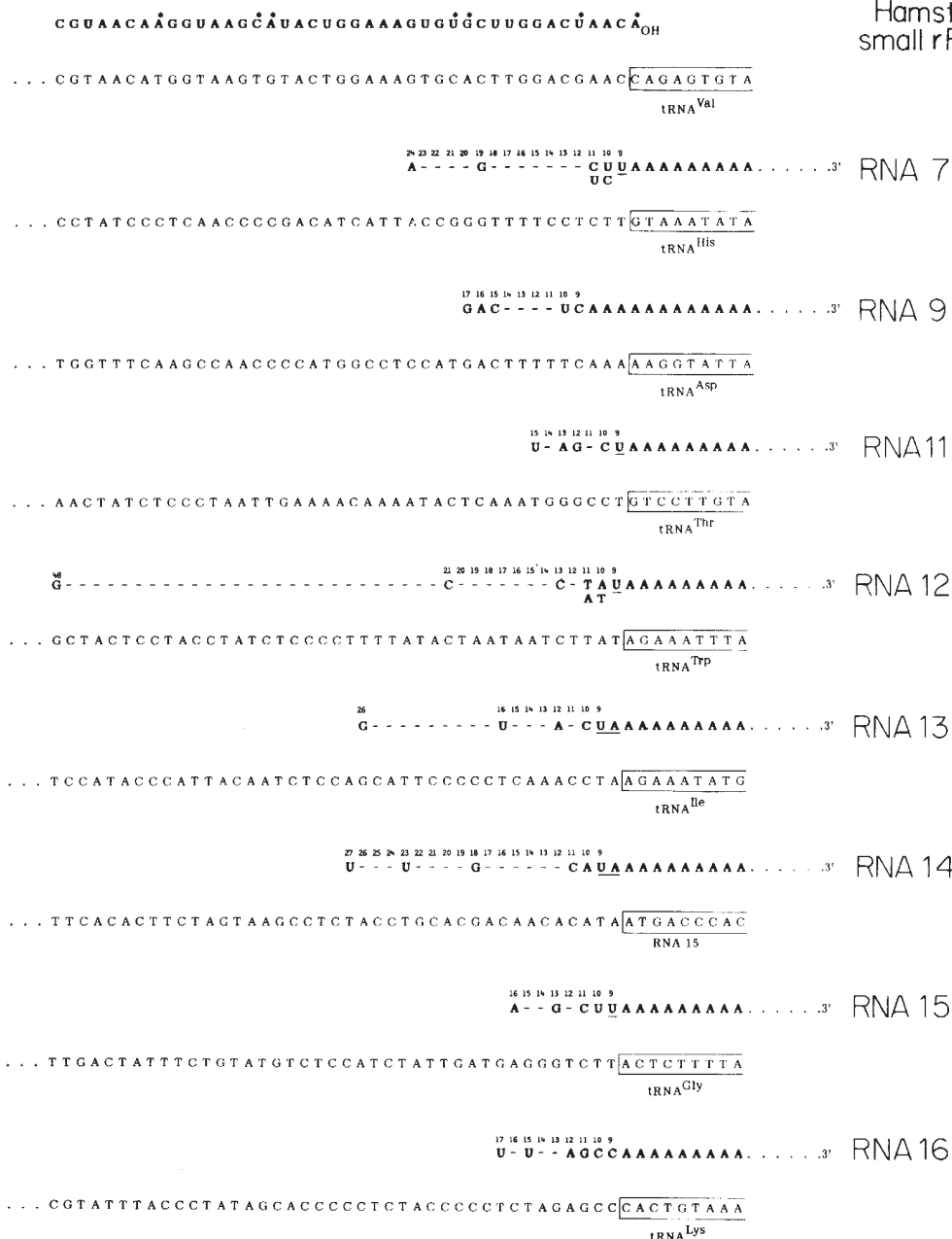


Fig. 3 Partial sequencing data determined for the 3'-end regions of the mitochondrial poly(A)-containing RNAs are shown aligned with the corresponding DNA sequences. A 5'-proximal segment of the sequence of the adjacent tRNA genes and of the RNA 15 coding stretch is also shown. The figure also includes the 3'-end proximal sequence of the hamster small mitochondrial rRNA¹³, together with the corresponding human mtDNA sequence and a portion of the contiguous tRNA^{Val} coding sequence. Asterisks indicate the non-homologous nucleotides.

Activation of a cellular *onc* gene by promoter insertion in ALV-induced lymphoid leukosis

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Analyses of DNA and RNA from avian leukosis virus (ALV)-induced lymphomas have provided strong evidence that, in most tumours, ALV induces neoplastic disease by activating the c-myc gene, the cellular counterpart of the transforming gene of MC29 virus. The data indicate that, as a rare event, the ALV provirus integrates adjacent to the c-myc gene and that transcription, initiating from a viral promoter, causes enhanced expression of c-myc, leading to neoplastic transformation.

RNA TUMOUR VIRUSES include acute transforming viruses (sarcoma and acute leukaemia viruses) and slow transforming viruses (for example, ALVs and murine leukaemia viruses). The mechanism by which the slow transforming viruses induce neoplastic disease has, until recently, remained obscure. These viruses differ from the acute transforming viruses in three important properties: (1) they induce macroscopic neoplasms in infected animals only after a latent period of 4–12 months, compared with 2–3 weeks for the acute viruses; (2) they do not induce morphological transformation at detectable frequency in tissue culture cells; and (3) they do not seem to contain a transforming gene.

ALVs cause B-cell lymphomas and occasionally fibrosarcomas, nephroblastomas, erythroblastosis, osteopetrosis, and anaemia. Recent evidence suggests that these viruses cause neoplastic transformation by activating a normal cellular gene(s). (1) Many ALV-induced lymphomas do not contain viral 35S and/or 21S mRNAs^{1,2} and many of the integrated proviruses in these tumours are defective^{1–3}. Thus it seems unlikely that a viral gene product is required for maintenance of neoplastic transformation. (2) The oncogenic potential of ALV seems to reside within the 3' portion of the viral genome—within ~500 nucleotides from the poly (A) tract^{4–6}—a region which does not seem to encode a viral protein⁷. (3) ALV-induced lymphomas contain proviruses integrated at one or more sites. However, at least some proviral information in each tumour is found integrated at one of a limited number of common sites^{1–3}. As proviral integration is known to occur at a large number of sites on the host chromosome, possibly at random^{8–12}, this apparent specificity of integration in lymphomas suggests that neoplastic transformation requires integration adjacent to a specific gene(s). (4) Primary and metastatic tumours are clonal^{1–3,13}. Metastases in the same bird contain similar or identical patterns of proviral integration, based on restriction analyses of cellular DNA; thus, transformation is a rare event which generally occurs only once in each animal. (5) ALV-induced lymphomas and lymphoma-derived cell lines contain new tumour-specific RNAs consisting of viral 5'-terminal sequences covalently linked to cellular sequences^{1,2}. These RNAs fall into a limited number of size classes¹. (6) The tumour-specific RNAs are expressed at moderately high levels (100–300 copies per cell)¹.

Based on certain structural features of the integrated ALV provirus, we^{1,14} and others^{2,4,6,11} have suggested a model for oncogenesis consistent with all the above observations, which we have termed oncogenesis by promoter insertion (see Fig. 1). Studies in many laboratories have shown that the integrated provirus consists of the viral structural genes flanked by sequences of ~350 nucleotides, termed long terminal repeats (LTRs)^{15–18}. The LTR contains a putative promoter sequence^{7,12,19,20}. Initiation of normal viral RNA synthesis occurs within the left LTR. Because this sequence is repeated at the right end of the provirus, initiation could also occur within

the right LTR. If the provirus integrated adjacent to a potentially oncogenic cellular gene, transcription initiated from the viral promoter could generate an RNA molecule such as those found in ALV-induced lymphomas, containing both viral and cellular sequences (see Fig. 1). The resultant enhanced expression of this cellular gene would lead to neoplastic transformation. Identification of the putative cellular gene activated by this mechanism would be strong evidence in support of this model.

Over 10 different 'transforming genes' have been identified in the genomes of acute transforming retroviruses of avian and mammalian origin^{21–26}—each of these viral *onc* (*v-onc*) genes has a normal cellular counterpart (cellular *onc*, or *c-onc*)^{24,27–34}. These normal cellular genes are presumably the progenitors of the *v-onc* genes and are generally expressed at low levels in normal, uninfected cells^{29,35–38}, although they may be expressed at higher levels during certain stages of development³⁸. As they are highly conserved in vertebrate evolution^{25,29,39}, these normal cellular genes probably have some essential role in cellular growth, differentiation and/or development.

It seemed possible that the normal cellular gene(s) activated by promoter insertion might be one of the cellular counterparts of a *v-onc* gene. Here we report our efforts to identify the putative *c-onc* gene(s) involved in ALV-induced lymphomas, using cDNA probes specific for five *v-onc* genes of avian acute

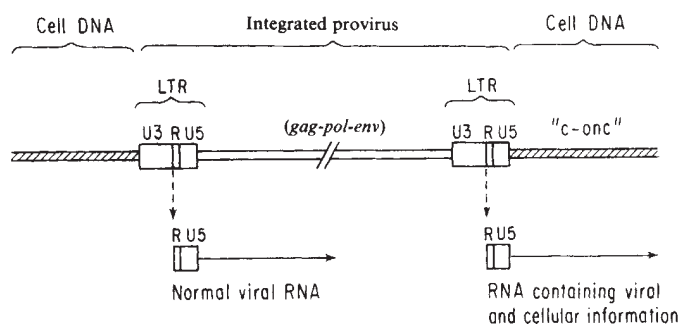


Fig. 1 Structure and transcriptional products of the integrated ALV provirus. The integrated provirus is flanked by sequences of ~350 nucleotides termed long terminal repeats (LTRs)^{15–18}. The LTRs, shown greatly enlarged to emphasize structural details, are composed of sequences derived from the 5' (U5) and 3' (U3) ends of genomic RNA, and a short sequence (R) of ~20 nucleotides, present at both ends of genomic RNA. Synthesis of normal viral RNA (genomic RNA and mRNAs) initiates within the left LTR. Initiation within the right LTR would generate a molecule containing viral 5' sequences (R + U5) plus cellular information encoded in the adjacent cellular DNA. If, as shown, the provirus integrated upstream from a potentially oncogenic cellular gene (designated *c-onc*), initiation within the right LTR could cause elevated expression of the *c-onc* gene.

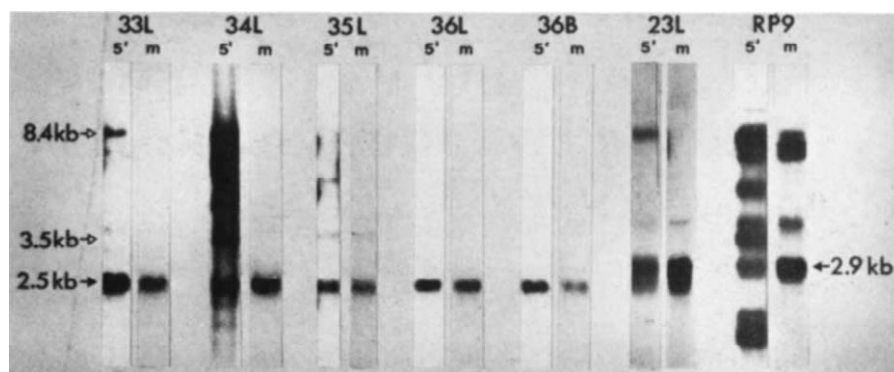


Fig. 2 *myc*-specific and viral 5'-specific RNAs in ALV-induced lymphomas. Poly(A)₁-containing RNAs (2–5 µg per lane) were glyoxylated, size-fractionated on 1% agarose gels, and transferred to aminophenylthioether paper by the procedure of Alwine *et al.*⁴³, as modified by B. Seed (personal communication). Details of this procedure have been described elsewhere¹. RNAs containing viral 5'-specific and *myc*-specific sequences were identified by hybridization to ³²P-labelled cDNA_s (5') and cDNA_{rep} (m) (specific activity ~2–4 × 10⁸ c.p.m. per µg). The blot was first hybridized to cDNA_s and exposed to X-ray film; it was then washed in denaturing conditions to remove the cDNA probe, and re-hybridized to cDNA_{rep}. Positions of viral 35S (8.4 kilobases) and 21S (3.5 kilobases) RNAs are indicated by open arrows; 2.5- and 2.9-kilobase *myc*-specific RNAs are indicated by closed arrows. Tissues are designated as in Tables 1 and 2. kb, Kilobase.

transforming viruses. We used three criteria to identify such a gene, based on predictions of the model for oncogenesis described above: (1) the *c-onc* gene should be expressed at elevated levels in ALV-induced lymphomas; (2) the *c-onc* sequences should be encoded in the tumour-specific RNAs, covalently linked to viral sequences; (3) proviruses in ALV-induced lymphomas should be integrated adjacent to the *c-onc* gene. Based on all three criteria, the data strongly suggest that most of the ALV-induced lymphomas studied result from activation of the *c-myc* gene, the cellular counterpart of the transforming gene of MC29 virus, by a promoter insertion mechanism.

Expression of *c-onc* genes in normal and neoplastic tissues

RNA from normal tissues and ALV-induced lymphomas was analysed by nucleic acid hybridization to cDNA probes specific for the following *v-onc* genes: *src* (Rous sarcoma virus), *erb* (avian erythroblastosis virus), *myb* (avian myeloblastosis virus), *fps* (Fujinami sarcoma virus) and *myc* (myelocytomatosis virus-29, or MC29). The cellular homologues of the five *onc* genes were expressed at low but detectable levels (1–4 copies per cell) in normal tissues from 3–4-month-old uninfected birds (Table 1). With one exception (see below), the cellular *src*, *erb*, *myb* and *fps* genes were expressed at similarly low levels in lymphoma-tous tissues.

In contrast, levels of *myc*-specific RNA were 30–100-fold higher in ALV-induced lymphomas (90–330 copies per cell). These data suggest that *c-myc* is the cellular gene responsible for ALV-induced neoplastic transformation in these tumours. An even higher level of *myc* RNA was found in RP9 (1,000 copies per cell), a cell line derived from an ALV-induced lymphoma. In RP9 cells, *myb*-specific RNA was also elevated, but to a much lesser extent (40 copies per cell); the significance of this is unclear.

A more extensive analysis of *myc* gene expression involving a larger number of normal tissues and ALV-induced lymphomas is shown in Table 2. Each tissue was also analysed for expression of virus-specific information by hybridization to a probe representative of the entire viral genome (cDNA_{rep}), and to a probe corresponding to the 5'-terminal 101 nucleotides of viral RNA (cDNA_s); the latter would detect 5'-terminal (R+U5) sequences of RNAs initiated from either the left or the right LTR (see Fig. 1). As 5'-terminal sequences comprise only ~1% of the viral genome, the presence of viral 5' sequences alone would not be detected by cDNA_{rep}. For detection by cDNA_{rep}, an RNA molecule must contain other viral genetic information.

Six main points can be drawn from the data in Table 2: (1) 12 of 14 ALV-induced lymphomas (including the lymphoma cell line RP9) contained elevated levels of *myc* RNA (at least 80 copies per cell). One additional lymphoma (29L) contained a slightly elevated level (9 copies per cell), whereas sample 25L contained only normal levels of *myc* RNA (2 copies per cell). (2) Uninfected chicken embryo fibroblasts and bursal and muscle tissues from 3–6-month-old uninfected birds contained low levels of *myc*-specific RNA (2–5 copies per cell). (3) In infected,

but non-neoplastic tissues (5B, 6B, 19M, 23M), *myc*-specific RNA levels were similar to those in tissues from uninfected birds. Viral RNA is present at elevated levels in these tissues. Thus it is clear that viral infection and/or expression of viral genes alone is not sufficient to induce *myc* expression. (4) Viral gene expression is not necessary for maintenance of neoplastic transformation. In ~50% of the tumours, cDNA_{rep}-specific RNA was present at levels (one to six copies per cell) only slightly higher than those of the endogenous provirus ev1⁴⁰ in normal cells⁴¹. (Most uninfected chicken cells contain genetic information homologous to the replicative genes of RNA tumour viruses. These 'endogenous proviruses' (ev) segregate as defined genetic loci.) (5) In each of the samples containing low amounts of RNA homologous to cDNA_{rep} (7K, 10B, 23L, 25L, 35L, 36B, 36L), viral 5'-specific sequences were present at elevated levels. In all these tumours (except 25L, in which *myc* gene expression is not elevated) the level of 5'-specific RNA was similar to that of *myc*-specific RNA. This is consistent with the interpretation that 5' and *c-myc* sequences are present on the

Table 1 Transformation-specific RNAs in ALV-induced lymphomas

Sample no.	Tissue	RNA (copies per cell)				
		<i>src</i>	<i>fps</i>	<i>erb</i>	<i>myb</i>	<i>myc</i>
12B	Uninfected	3	1	2	3	4
31L	Uninfected	2	1	4	1	2
7K	Lymphoma	4	1	6	5	110
11B	Lymphoma	3	2	2	4	180
23L	Lymphoma	5	1	2	3	330
9B	Lymphoma	5	1	4	2	230
22L	Lymphoma	4	2	ND	5	90
RP9	Lymphoma cell line	3	1	3	40	1000

Sample designations indicate the animal number and the source of the tissue: B, bursa; L, liver; K, kidney. RP9 is a continuous cell line derived from a RAV-2-induced lymphoma (provided by Dr W. Okazaki). Lymphomas were removed from leukotic birds 4–6 months after infection with ALV. Birds were injected with 10⁶–10⁷ plaque-forming units (PFU) of virus, 2–7 days after hatching. Virus strains used in this and other experiments included Rous-associated virus-1 (RAV-1), RAV-2 and transformation-defective (td) mutants of Schmidt-Ruppin Rous sarcoma virus-A (td107A, td103 and td109). No virus strain-specific differences were observed in any of the experiments described here. Normal tissues were taken from uninfected 3–6-month-old birds. Hybridizations were performed in liquid as described elsewhere³⁵, using ~5 mg ml⁻¹ of cellular RNA. Hybrid reactions were stopped at various time intervals, corresponding to C_tt values of ~10–10³ mol·s⁻¹. RNA concentrations were calculated from C_tt values (see ref. 35), assuming that a C_tt value of 4 × 10⁴ mol·s⁻¹ is equivalent to one copy per cell. Because of variations in the RNA content of cells from different tissues, the values shown are not precise but the error introduced is no more than a factor of 2–3, and thus does not affect any of the conclusions drawn. Radiolabelled cDNA probes correspond to the unique (putative transforming) sequences of the following acute transforming viruses^{24,27,61,62}: Rous sarcoma virus (*src* gene), Fujinami sarcoma virus (*fps*), avian erythroblastosis virus (*erb*), avian myeloblastosis virus (*myb*), and myelocytomatosis virus-29 (*myc*). Probes were prepared as previously described for cDNA_{src} (ref. 35), except that cDNA was synthesized from purified viral RNA and reverse transcriptase, using random DNA primer⁶³. Sequences unique to each of the acute transforming viruses were selected by removing sequences common to ALV (by hybridization to a mixture of ALV RNAs from RAV-1, RAV-2, RAV-61, plus erythroblastosis-associated virus for cDNA_{src}, and myeloblastosis-associated viruses-1 and -2 for cDNA_{myb}). The probes demonstrated no cross-homology, and did not hybridize (<2%) with any of the ALV RNAs used for their selection, or to ribosomal RNA. ND, not determined.

same RNA molecules in ALV-induced lymphomas. (6) *c-myc*-specific RNA in normal cells does not contain viral 5' sequences—the latter were undetectable (less than 0.1 copy per cell) in tissues from an ev-negative bird (37L and 37B)⁴², whereas *myc*-specific RNA was present at 2 and 5 copies per cell, respectively.

Tumour-specific RNAs have *myc*-specific sequences and viral 5' sequences

To test directly whether the *myc*-specific sequences and viral 5'-sequences are encoded on the same RNA transcript, poly(A)-containing RNA from ALV-induced lymphomas was analysed by the 'Northern' transfer technique⁴³, using cDNA_{5'} and cDNA_{myc} as probes. Previous studies showed that almost all tumours synthesize discrete new tumour-specific RNAs containing viral 5' sequences plus other information, presumably derived from the cell¹. In most tumours, the size of these RNAs was ~2.5 kilobases, although a few contained RNA of ~2.9 kilobases. None of these RNAs contained sequences detectable with cDNA_{rep}. In a few tumours, larger RNAs of 5–6 kilobases were detected with cDNA_{5'}. These RNAs also contained information detectable with cDNA_{rep}.

Six of the seven samples shown in Fig. 2 contained a single major *myc*-specific RNA. In each case, this RNA corresponded to a species detectable with cDNA_{5'}, suggesting that the *myc* sequences and viral 5' sequences are present on the same RNA transcript. As reported previously¹, these RNAs are not detected with cDNA_{rep}. In five tumours, the size of this RNA was 2.5 kilobases, whereas in a sixth (23L), it was rather heterogeneous, with a size of ~2.9 kilobases. A 2.9 kilobase RNA was also found in RP9 cells, which also contained four additional *myc*-specific RNA species, each of which corresponded to a 5'-containing RNA. The three larger of these, with sizes of 7–8.5 kilobases, were rather obscured in the cDNA_{5'}-specific analyses by the presence of normal viral 35S RNA, but were seen clearly in short exposures of the blots. The high degree of specificity of the cDNA_{myc} probe can be seen from the fact that this probe did not hybridize to the 35S viral RNA in tumour 34L, although this species is much more abundant than the 2.5-kilobase RNA detected with cDNA_{5'} and cDNA_{myc}. RP9 and 34L also contain several smaller RNAs that hybridize with cDNA_{5'} and cDNA_{rep} (ref. 1) but not with cDNA_{myc}; these RNAs are presumably aberrant viral transcripts.

myc-specific RNA was not detected in normal cells under the conditions used in these experiments (data not shown). Sheiness *et al.*⁴⁴ have identified *c-myc* RNA in uninfected cells using larger amounts of cellular RNA. These workers reported a size of 2.8 kilobases, slightly larger than that of the most frequently observed *myc*-specific RNA found in ALV-induced lymphomas.

Genetic content of tumour-specific RNAs

The data described above are consistent with the interpretation that the *myc*-specific RNAs in ALV-induced lymphomas have the structure illustrated in Fig. 1 (that is, they contain only ~100 nucleotides of virus-coded information). The failure to detect these RNAs with cDNA_{rep} (ref. 1) suggests that they contain little or no viral information other than the 5'-terminal sequences detected with cDNA_{5'}. As analysis with cDNA_{rep} does not provide a sensitive assay for possible short stretches of viral information, RNA from ALV lymphomas was tested further by liquid hybridization, using probes specific for portions of the viral genome.

The low level of normal viral mRNAs in some of the tumours allowed us to examine the genetic content of the *myc*-specific RNA in these tissues. Two tumours, 7K and 23L, in which *myc*-specific RNA was at least 50-fold more abundant than viral mRNA (Table 2), were chosen. RNA from these tumours was hybridized to cDNA_{myc} (Fig. 3, broken lines) and to probes specific for the viral *gag*, *pol*, and *env* genes, the 500 nucleotides adjacent to the poly(A) tract at the 3' end of the viral genome

(cDNA_{3'}), and cDNA_{5'} (solid lines). With both tumours, cDNA_{myc} hybridized to a plateau level of ~85%, indicating extensive homology between the *myc* probe and the *c-myc* sequences encoded in the tumour-specific RNA of ALV-induced lymphomas. A plateau value of ~90% hybridization was obtained with RNA from normal cells, when hybridization was carried out to very high *C_t* values (data not shown).

Of the virus-specific probes, only cDNA_{5'} hybridized with kinetics similar to those obtained with cDNA_{myc}. Some hybridization was obtained with the other probes at much higher *C_t* values. This could reflect the presence of small amounts of viral mRNA. However, in no case were the hybridization patterns consistent with the presence of any viral sequences, other than 5'-specific sequences, in the *myc*-specific RNA transcript. Thus we conclude that, within the limits of this assay (probably ~5% of the genetic content of each probe), no viral information other than 5' sequences is present in the *myc*-specific transcript of tumours 7K and 23L. Hybridizations of gene-specific probe to the 'Northern' blots similarly failed to detect other viral sequences on the 2.5- and 2.9-kilobase *myc*-specific RNAs (data not shown).

ALV provirus is integrated adjacent to *c-myc* in ALV-induced lymphomas

In our previous studies¹, analysis of lymphoma DNA was performed by digestion with the restriction endonuclease *EcoRI*, which cuts at several sites within the provirus^{15,16,45}. The most useful restriction fragment generated by this digestion is the right-end 'junction fragment', which contains ~150 nucleotides of viral information, plus adjacent downstream cellular DNA. If the ALV provirus is integrated adjacent to *c-myc*, this junction

Table 2 *myc*-specific and virus-specific RNA in normal and neoplastic tissues

Sample no.	Tissue	RNA (copies per cell)		
		<i>myc</i>	5'	<i>rep</i>
2928F	Normal (uninfected)	2	0.8	0.7
31L	Normal (uninfected)	2	0.6	0.5
12B	Normal (uninfected)	3	0.7	0.7
37L	Normal (uninfected)	2	<0.1*	<0.1*
37B	Normal (uninfected)	5	<0.1*	<0.1*
19M	Normal (infected)	2	1,500	1,300
23M	Normal (infected)	3	1,500	1,200
5B	Normal (infected)	3	145	160
6B	Normal (infected)	2	200	280
3B	Pre-neoplastic bursa	10	7	0.4
7K	Lymphoma	110	100	2
9B	Lymphoma	230	1,300	1,100†
10B	Lymphoma	100	90	2
11B	Lymphoma	180	2,500	2,000
19L	Lymphoma	85	1,900	1,300
22L	Lymphoma	90	300	300
23L	Lymphoma	330	300	5
25L	Lymphoma	2	90	2
29L	Lymphoma	9	1,700	1,300
34L	Lymphoma	105	1,000	800
35L	Lymphoma	80	80	6
36B	Lymphoma	100	100	2
36L	Lymphoma	140	120	3
RP9	Lymphoma cell line	1,000	4,000	600

Sample designations are as in Table 1; F, fibroblasts (chicken embryo); L, liver; B, bursa; M, muscle; K, kidney. Tissues were taken from 3–6-month-old uninfected birds, or from leukotic infected chickens 4–6 months after infection (see Table 1 for details). RNA concentrations were determined as in Table 1. RNA was detected with cDNA_{myc}, or with virus-specific probes corresponding to 5'-terminal sequences (R+U5) of genomic RNA (cDNA_{5'}) or to the entire viral genome (cDNA_{rep}). cDNA_{5'} ('strong-stop DNA'⁶⁴) was synthesized from RAV-2 as described elsewhere¹; cDNA_{rep} was synthesized from RAV-2 RNA, using random DNA primer⁶². cDNA_{5'} detects RNA transcripts initiating either from the left or right LTR (see Fig. 1), whereas cDNA_{rep} detects only viral RNA containing substantial portions of the viral coding sequences (presumably initiating from the left LTR).

* No virus-specific RNA was detected with either cDNA_{5'} or cDNA_{rep} in conditions that would have detected 0.1 copies per cell. These samples were taken from a chicken (no. 37) which lacks endogenous proviruses⁴².

† RNA from tumour 9B hybridized with kinetics suggesting two components, with levels of 25 and 1,100 copies per cell. Other experiments demonstrate the presence in this tumour of an abundant 5.5-kilobase RNA species containing *gag* and 3' viral sequences, and portions of *pol*.

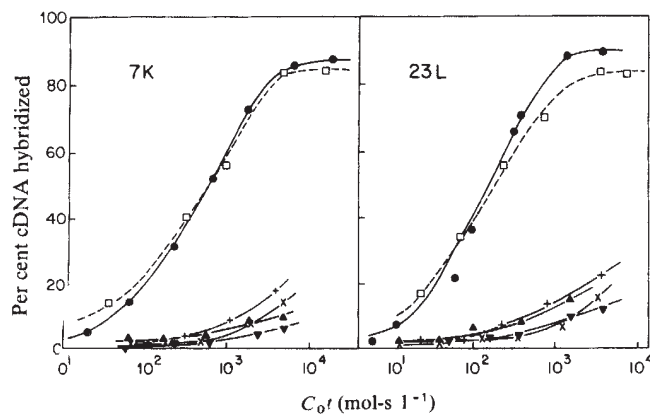


Fig. 3 Genetic content of *myc*-specific RNAs in two ALV-induced lymphomas. Total cellular RNA from tumours 7K and 23 L was extracted with proteinase K-phenol-chloroform³⁵, and precipitated two or three times with 2 M LiCl to remove cellular DNA. RNA was hybridized to cDNA_{myc} (broken line) and to cDNA probes specific for different portions of the ALV genome (solid lines). The following virus-specific probes were used: Δ , cDNA_{gag}; ∇ , cDNA_{pol}; $\times$, cDNA_{env}; $\bullet$, cDNA_{5'}; and $+$, cDNA_{3'}. cDNA_{pol} was prepared from RAV-0, and corresponds to the deletion in ev3 RNA⁴¹, which includes a large portion of the *pol* gene, and the 3' portion of *gag*. All other probes were prepared from RAV-2. cDNA_{gag} represents the 5' 2,500 nucleotides of genomic RNA, and overlaps somewhat with cDNA_{pol}. cDNA_{env} corresponds to the *env* deletion of Bryan strain of Rous sarcoma virus, and cDNA_{3'} corresponds to the 3' 500–600 nucleotides ('common' or 'c' region) of genomic RNA. cDNA_{5'} is described in Table 2 legend. Probes were prepared, and hybridizations performed as described elsewhere^{35,41}.

fragment should contain *myc*-specific sequences, and thus be detected with both cDNA_{5'} and cDNA_{myc}.

Restriction analyses of DNA from normal tissue and from three ALV-induced lymphomas are shown in Fig. 4. In normal cells, *c-myc* sequences were contained almost entirely within a single *Eco*RI restriction fragment of ~14 kilobases, consistent with the report of Sheiness *et al.*⁴⁴. (We also found a second, minor band at ~2 kilobases which has not been further characterized.)

Lymphoma DNAs contained a new *myc*-specific restriction fragment not present in normal cells (Fig. 4). In each case, this new fragment corresponded to a junction fragment detected with cDNA_{5'}. This presumably represents integration of an ALV provirus adjacent to the *c-myc* gene. In a larger sampling of lymphomas, we found that 31 out of 37 lymphoma DNAs contained proviral information integrated adjacent to *c-myc*⁴⁶. Those samples that did not contain elevated levels of *myc*-specific RNA also did not contain proviruses integrated adjacent to *c-myc*. The 14- (and 2-) kilobase *myc*-specific *Eco*RI fragments found in normal cells were also found in the lymphomas. This would be expected because integration presumably occurs adjacent to only one of the copies of *c-myc* present in the diploid chicken genome.

We have recently succeeded in cloning the *Eco*RI junction fragments from two ALV-induced lymphomas (B.G.N. *et al.*, unpublished results). The cloned DNAs contain both viral 5' information and *myc*-specific information, thus providing direct evidence that the viral and *c-myc* sequences are covalently linked in these tumours.

Discussion

From the above data we conclude that, in almost all ALV-induced lymphomas, neoplastic transformation results from activation of the cellular gene *c-myc*. This process seems to involve integration of the provirus upstream from the *c-myc* gene, and transcriptional activation of this gene due to the insertion of the 'strong' viral promoter. The resultant RNA transcript contains both viral and *c-myc* information, and is present at levels 30–100-fold higher than that of *c-myc* RNA in normal tissues.

Integration of the provirus at a site which could activate the *c-myc* gene would presumably be a rare event, because integra-

tion is known to occur at many sites (possibly at random) in the host genome^{8–12}. Thus, this mechanism can explain, at least in part, the long latent period required for development of lymphomas in ALV-infected birds, and the failure to detect transformation in ALV-infected tissue culture cells.

We have previously observed that ALV-induced lymphomas contained proviruses integrated at a limited number of discrete sites, as judged by restriction analysis. The junction fragments of different size observed in the earlier experiments could represent ALV integrations adjacent to different cellular genes. However, our data (see Fig. 4 and ref. 46) indicate that most proviruses are integrated adjacent to the same gene, *c-myc*. Apparently, integration at several locations upstream from *c-myc* can lead to activation of this gene. Many tumours that have junction fragments of different sizes (for example, 34L and 35L) synthesize *myc*-specific RNAs of the same size (2.5 kilobases). This could be explained if the 2.5-kilobase RNAs arose by splicing events, with removal of varying lengths of intervening sequences.

It is unclear why *c-myc* should be involved in such a large percentage (~85%) of lymphomas. Over 10 transforming genes (*v-onc*), each of which has a cellular counterpart that is highly conserved in all vertebrates, have been identified in different avian and mammalian retroviruses^{21–34}—others undoubtedly exist. Although some *v-onc* genes may show tissue specificity, this alone cannot explain the preferential involvement of *c-myc* in B-cell lymphomas. MC29, an acute transforming virus which contains the *myc* gene, causes myelocytomatosis, fibrosarcomas and carcinomas in infected birds⁴⁷. No examples of B-cell lymphomas have been reported. In tissue culture, MC29 transforms both haematopoietic cells⁴⁸ and fibroblasts^{47,48}. However, other viruses (for example, AMV and AEV) also transform haematopoietic cells *in vivo* and *in vitro*²³. Nevertheless, the cellular counterparts of these *v-onc* genes are not activated by ALV in a large percentage of ALV-induced lymphomas.

Breindl *et al.*⁴⁹ have suggested that proviruses integrate preferentially into transcriptionally active regions of chromosomes. If *c-myc* is in a transcriptionally active conformation at some stage of B-cell differentiation, integration within this region might occur with a higher than random frequency. Alternatively, *c-myc* may be more readily activated by the promoter insertion mechanism, due to some structural feature(s) of this

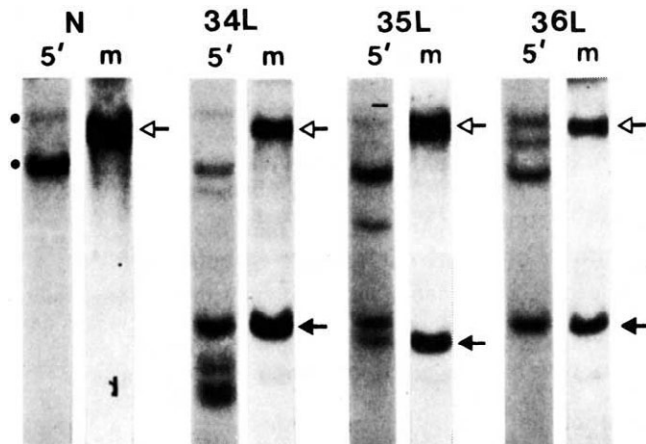


Fig. 4 Restriction analysis of *myc*-specific and viral 5'-specific sequences in DNA from normal cells and ALV-induced lymphomas. DNA was extracted from normal brain (N) from bird no. 34, and from three ALV-induced lymphomas (designated as in Tables 1 and 2). DNA was digested with restriction endonuclease *Eco*RI, size-fractionated on agarose gels and transferred to nitrocellulose paper by the technique of Southern⁶⁵. Experimental details have been described elsewhere^{1,40}. Duplicate samples from each tissue were analysed in parallel experiments by hybridization to cDNA_{5'} (5') or cDNA_{myc} (m). Two *myc*-specific restriction fragments—a major species of 14 kilobases (open arrows) and a minor species of 2 kilobases—were detected in all DNAs from both normal and neoplastic tissues. *myc*-specific fragments of 2.9 and 2.7 kilobases (solid arrows), also detected with cDNA_{5'}, were found only in tumour DNAs. cDNA_{5'}-specific restriction fragments of the endogenous provirus cv1.30 (indicated in the left lane by solid circles) were present in both normal and transformed tissues.

gene. As discussed above, integration at several sites upstream from *c-myc* can apparently result in its activation. This multiplicity of potentially 'productive' sites might increase the probability of activating this gene over other *c-onc* genes, which might only be activated by integration at a more limited number of sites.

It is possible that other *c-onc* genes are activated by promoter insertion in the ALV-induced lymphomas that do not show enhanced *myc* expression. We are currently attempting to identify the cellular gene(s) induced in these tumours.

We could find no evidence for the participation or generation of an acute transforming virus in ALV-induced lymphomas; no virus capable of transforming fibroblasts was recovered from any of the lymphomas tested (data not shown). (MC29 transforms fibroblasts in tissue culture with high efficiency.) Virus from one sample, RP9, was further tested by injection into 1–7-day-old chicks. Lymphomas appeared after a long latent period (>4 months), but no acutely developing neoplasia was observed. In addition, the structure of the *myc*-specific RNAs in lymphomas was not consistent with that of a viral genome. All known defective transforming viruses contain viral sequences at the 5' and 3' ends of their genome. Sequences at the 3' end encode recognition signals for reverse transcription of viral RNA, integration of proviral DNA and synthesis of viral mRNA. Analyses of RNA from two ALV-induced lymphomas (Fig. 3) revealed no viral 3' information. Furthermore, sequences thought to be involved in RNA packaging⁵⁰, located in the region from ~100–350 nucleotides from the 5' end⁵¹, do not seem to be present in the tumour-specific RNAs.

Cooper and Neiman⁵² using a transfection assay, recently demonstrated that DNA from ALV-induced lymphomas causes transformation of NIH/3T3 cells with high efficiency. Surprisingly, the transformants did not contain any detectable viral DNA sequences (including the LTR), nor apparently did they contain *myc*-specific sequences derived from the lymphoma DNAs. However, these investigators have recently found that the lymphomas from which the DNA was derived do contain ALV proviruses integrated adjacent to *c-myc* (G. Cooper and P. Neiman, personal communication). The transfection assay is apparently detecting another genetic change in lymphomas which may be a direct or indirect consequence of enhanced *c-myc* expression. It is possible that *c-myc* activation, and the activity detected in the transfection assay, represent different steps in a multistage process leading to neoplastic transformation.

The *onc* gene of MC29 virus^{53–55}, and those of many other acute transforming viruses⁵⁶, are expressed as *gag-onc* fusion proteins. Our data indicate that the *gag*-encoded portion of this protein is not required for the transforming activity of the *myc* gene product at least in B cells. We could detect no viral sequences other than the 5'-terminal 100 nucleotides in *myc*-specific RNA from these tumours; thus the predicted translation product of this RNA would lack *gag* sequences.

Earlier work had suggested that normal cellular information might be potentially oncogenic. Hanafusa *et al.*⁵⁷ generated acute transforming viruses (recovered avian sarcoma viruses, rASVs), by injecting transformation-defective viruses which lack most, but not all, of the *src* gene, into chickens. The rASVs have acquired most of their *src* information from the cell by recombination with *c-src*^{58–60}. These experiments suggested that *c-src*, without substantial modification, is capable of inducing cell transformation when expressed at high levels as a consequence of its incorporation into the viral genome. Recently, Oskarsson *et al.*³³ have shown that *c-mos*, the cellular counterpart of the transforming gene of Moloney murine sarcoma virus, can induce transformation of NIH/3T3 cells if the LTR is linked to cloned *c-mos* DNA. The resultant structure is similar to that found in ALV-induced lymphomas. No viral sequences other than the LTR are required for this activity (G. Vande Woude, personal communication).

It seems likely, therefore, that the role of both acute and slow RNA tumour viruses in neoplastic transformation is simply to provide a means for constitutively expressing a cellular gene that is normally expressed at low levels. These viruses accomplish this in slightly different ways. In the acute viruses, a cellular gene has been inserted into the viral genome (by recombination), where it comes under the control of the viral promoter. With slow transforming viruses such as ALV, the viral promoter is inserted into (or adjacent to) the cellular gene.

The data presented here show, for the first time, that a carcinogenic agent (ALV) acts by altering the expression of a normal cellular gene. These findings raise the interesting possibility that activation of a *c-onc* gene may be the common initiation event in neoplastic transformation by both viral and non-viral agents. Radiation and nearly all chemical carcinogens are potent mutagens—these might induce elevated expression of a *c-onc* gene by altering regulatory sequences (by point mutation or DNA modification), and/or by causing translocations that join *c-onc* coding sequences to transcriptionally active elements. The different types of cancer may reflect the particular *c-onc* gene activated, and the type of cell in which activation occurs. The availability of molecular probes for the known *onc* genes should allow screening for enhanced expression of these genes in a large number of tumours of unknown origin. Thus it should be possible to test the general validity of this model.

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LETTERS

A companion quasar to 3C345

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Although some quasistellar objects (QSOs) are known to be members of small groups of galaxies (see refs 1–3), there is no known case of a QSO in a rich cluster of galaxies⁴. Whether this has important implications for the nature of QSOs, or is simply a reflection of the difficulty of observing high-redshift clusters, is unclear. Here we report the discovery of a new QSO which provides perhaps the strongest evidence to date for quasar membership in a rich cluster.

The QSO was discovered during a programme aimed at the identification of optical counterparts of X-ray sources observed serendipitously by the Einstein Observatory. The detector used, the imaging proportional counter⁵, yields positional accuracies of the order of 1 arc min, typically restricting optical candidates for the X-ray source to only a few objects. These candidates were then each examined spectroscopically. So far we have identified more than 40 previously uncatalogued QSOs using this technique, and preliminary results on the objects as a class are reported elsewhere⁶. During X-ray observations of quasar 3C345, a serendipitous X-ray source located 8 arc min north-east of the quasar was discovered, in addition to emission from 3C345 itself. The observed flux from the new source is 3.2×10^{-13} erg cm⁻² s⁻¹ in the 0.5–4.5-keV band. This flux, which is roughly 7% of that observed from 3C345 (ref. 7), is typical of the serendipitous sources in our sample. Three separate observations during a 6-month period in 1979–80 showed no evidence for intensity variability of the new source in excess of the $\pm 20\%$ statistical uncertainty in the count rate. There was also no evidence for X-ray variability in 3C345 during this period.

Spectroscopic observations of optical candidates in the X-ray field were obtained on 16 May 1980, using the Robinson-Wampler image tube scanner⁸ at the Lick Observatory 3-m Shane reflector; the grating yields 20 Å spectral resolution in the 3,500–8,500 Å range. The X-ray source was readily identified with a previously unreported faint QSO, which we designate 1641+3998. The coordinates of the object are $\alpha(1950) = 16$ h 41 min 54.1 s, $\delta = +39^\circ 59' 17''$. Based on calibrations of the image diameter of the object on the Palomar Observatory Sky Survey⁹, we estimate the magnitude of the object at the epoch of

those plates as $B = 18.7 \pm 0.5$. Both the Sky Survey and our spectrophotometry show the QSO to be quite blue. As stressed in ref. 6, the probability of a chance coincidence of a random QSO with the X-ray source is negligible, so the identification of the quasar with the source is secure. The finding chart for the object in Fig. 1 is reproduced from a 4-m Mayall telescope plate obtained in 1973; also shown is 3C345.

The spectrum of the newly discovered QSO is of particular interest, and is shown in Fig. 2. Despite the modest signal-to-noise ratio, four strong emission lines are clearly visible at $\lambda\lambda 4,450, 6,912, 7,750$, and $7,980$ Å. We identify these features as Mg II $\lambda 2,800$, H γ , H β and [O III] $\lambda 5,007$, respectively, and derive a galactocentric redshift $z = 0.594 \pm 0.001$, where the uncertainty is dominated by the breadths of the permitted emission lines. The galactocentric redshift of 3C345 (see refs 10, 11) is $z = 0.595$, where again we estimate a formal uncertainty of at least ± 0.001 due to emission line widths. Thus to within the measurement errors, the redshifts of these two quasars are identical. The spectra of the objects are at least superficially similar in appearance, although this is perhaps not surprising in view of the limited variety in the emission lines which are typically seen in QSOs at this redshift.

We may estimate the probability that the remarkable redshift agreement of the two objects is a chance coincidence of two randomly distributed, unrelated QSOs. Our X-ray observations⁶ yield 0.6 serendipitous sources per square degree, and we identify about half of these as QSOs with $z \leq 1$. Thus we expect to observe $\approx 6 \times 10^4$ QSOs before finding one with a

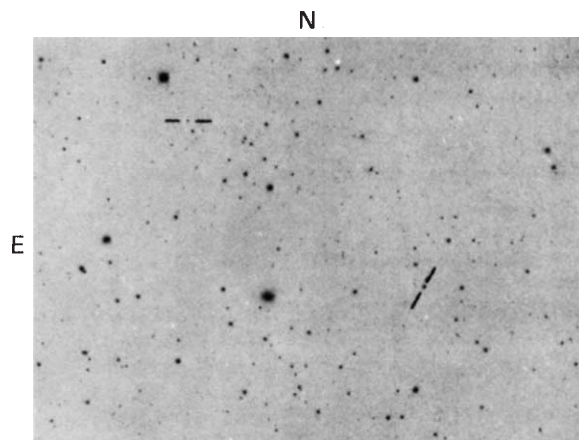


Fig. 1 A blue plate (IIa0 + GG385) of the field of the two quasars, obtained by Kinman with the Mayall 4-m telescope on 3 June 1973. The newly discovered quasar, 1641+3998, is the fainter of the two flagged objects, near the north-east of the field; 3C345 is the brighter object marked 8 arc min to the south-west.

'companion' as close in position (0.06 deg^2) and redshift (10^{-3}) as the current example is to 3C345. In fact only about 100 QSOs had been observed⁷ when 1641+3998 was discovered, so the probability of a chance coincidence is ~ 0.002 . Therefore, tempered by the hazards of *a posteriori* probability calculations, we conclude that the association of 3C345 and 1641+3998 is statistically significant and unlikely to be accidental.

If we accept the physical association of our two quasars, the explanations for such a situation are limited. We have briefly considered the extraordinary possibility that 1641+3998 could be a gravitationally focused image of 3C345, similar to the two currently known examples of this effect^{12,13}. The very large angular separation of the images compared with the previous cases would imply an unusual mass and/or mass distribution for the focusing matter. Remarkably this situation cannot be immediately dismissed, as a cluster of galaxies at $z = 0.01$ with mass $2 \times 10^{14} M_{\odot}$ could create such an image. However, such a cluster must have a very extreme mass-to-luminosity ratio to avoid obvious detection. A more awkward difficulty with this hypothesis is that the ratio of fluxes of the two QSOs should be independent of wavelength. Although the optical and X-ray flux ratios of the two objects do agree well (within 3 orders of magnitude), 3C345 is an intense radio source (11 Jy at 178 MHz) leading to the prediction of an easily detectable radio flux from 1641+3998, barring long differential time delays. Maps which subtract the side lobes of 3C345 (ref. 14) do not show such a source, to limits of 10 mJy. The agreement of the ratio of optical and X-ray fluxes is thus probably simply a manifestation of the relative constancy of this parameter previously noted in large samples of radio-selected¹⁵ as well as X-ray selected⁶ QSOs.

The alternative hypothesis, which we favour, is that the redshift agreement of these two QSOs indicates their common membership in a cluster of galaxies. The projected separation of several Mpc would then indicate this to be a rich cluster, the first such example of a cluster with QSOs. Although theoretical calculations of cluster diameter evolution with redshift¹⁶ indicate that distant rich clusters should be smaller than at the current epoch, this poses no difficulty in the present case as these evolutionary effects become noticeable only at larger redshift. As the velocity dispersion in rich clusters of galaxies is as large as $1,500 \text{ km s}^{-1}$, one might worry that the observed small limit on the velocity difference between 3C345 and 1641+3998 ($\leq 300 \text{ km s}^{-1}$) implies a cluster velocity dispersion grossly inconsistent with this norm. However, this proves not to be the case. If for simplicity, the radial velocities of the cluster galaxies are assumed to have a gaussian distribution, then we can convert the observed difference between two members into 90% confidence limits on the actual dispersion. A 300 km s^{-1} difference between two cluster members implies a cluster dispersion in the range $100\text{--}3,000 \text{ km s}^{-1}$ (90% confidence). Thus, perhaps surprisingly, the small observed velocity difference is not an awkward constraint on this model. The virial mass implied for the cluster by this allowed range of dispersions is in the range $10^{13.5}\text{--}10^{16.5} M_{\odot}$, again, not restrictive, but consistent with the parameters of known rich clusters.

Our failure to detect X-ray emission from this putative cluster is not necessarily surprising, but means that the cluster has an X-ray luminosity less than about half that of the Coma cluster (the X-ray bright core of which would appear point-like at this distance). However, Einstein observations (F. Abramopoulos, personal communication) indicate this is not unusual even for rich clusters. In addition, if 3C345 lies close to the centre of the cluster, then the possibility of source confusion implies that the cluster will be even more difficult to detect in X rays.

Our hypothesis can be tested in that the normal galaxies in this putative cluster are expected to appear at $V = 22\text{--}23$ and thus be directly observable. Good quality 4-m plates of the field obtained by Kinman do show numerous faint galaxies nearby. However, the space density of clusters at this faint apparent magnitude is large enough that two or three such clusters are expected on every high latitude 4-m plate¹⁷. Thus the prob-

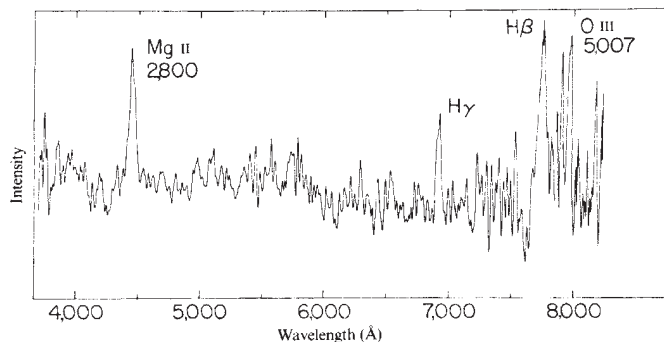


Fig. 2 The spectrum of the quasar 1641+3998, obtained with the Lick 3-m Shane reflector, with $20\text{-}\text{\AA}$ spectral resolution. The data have been converted to relative fluxes above the atmosphere through observation of spectrophotometric standard stars, and the most prominent emission features are identified.

ability of a chance superposition of a faint, unrelated cluster on this pair of QSOs is not negligible, and radial velocities of the galaxies will be necessary to verify the proposed association.

Attention has recently been drawn to other close groupings of QSOs with similar redshifts¹⁸⁻²², and diverse interpretations involving clusters, superclusters and non-cosmological redshifts have been advanced. Whether these pairs, including the one discussed here, represent clusters or superclusters is of secondary importance to the broader conclusion that QSOs exhibit similar clustering characteristics as galaxies. The pair discussed here, being at low redshift, should be suitable for future research.

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Random alignment of quasars

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Arp and Hazard¹ have reported several peculiar alignments of quasars, alignments which are exact to the limit of resolution on a Schmidt plate (2 arc s). We report here our investigation of the claim that the probability of such alignments occurring purely by chance is exceedingly small. Because the correct statistical analysis is not immediately obvious, we have performed a series of computer experiments to investigate how often such alignments will occur by chance.

Several trials were made in which 300 quasars were placed at random in two cartesian coordinates on a square, and each triplet of points whose overall linear extent did not exceed $1/7$ of the square side was examined for a possible alignment. Values of P , the deviation of the intermediate quasar from the line joining the other two members of the triplet, were determined. An example of the peculiar configurations that can arise by chance is shown in Fig. 1, taken from one of the trials.

The scale on the square depends entirely on the assumed density of quasars. Observational estimates² suggest that the density of quasars down to the twentieth magnitude is between 5 and 10 quasars per square degree. We have chosen a (possibly conservative) estimate of 6 quasars per square degree, (the value assumed by Arp and Hazard). On this scale, the whole square corresponds to approximately 50 square degrees of sky.

As the positions of all the quasars on the square (once placed) are known to very high accuracy, P is also known to high accuracy (corresponding to a resolution of a fraction of an arc s). In observations on a Schmidt plate, however, each image can be resolved only to 2 arc s, resulting in an uncertainty in alignment of somewhat more than 2 arc s. We have therefore chosen 2.4 arc s as a limit for P , below which we regard an alignment to be good. The number of alignments found (classified into various ranges of the value P up to 6 arc s) are shown in Table 1, the size

Table 1 The distribution of P (the displacement of the intermediate quasar of each triplet from a perfect alignment) for outer quasar separations $\leq 1^\circ$

P (arc s)	Mean of 16 runs	Largest no. in a single run	Smallest no. in a single run
0–0.24	1.6	4	0
0.24–1.20	7.4	15	4
1.20–2.40	7.9	14	4
2.40–3.60	7.4	19	2
3.60–4.80	9.4	16	5
4.80–6.00	8.2	12	5

limit on allowed alignments corresponding to 1° . Table 2 shows the number of alignments $P < 6$ arc s as a function of the separation d of the outer quasars of the alignment. χ^2 -tests indicate that these distributions are consistent with a uniform distribution in P , and a cubic distribution in d . Certain random alignments would, of course, run off the edge of the square, and thus be lost. This edge effect also occurs on Schmidt plates, but we believe that the proportion of alignments lost by this effect does not vary by more than about 10% between the square ($7^\circ \times 7^\circ$) and a typical Schmidt plate ($6^\circ \times 6^\circ$).

An estimate of alignment probabilities can be made by the following simple argument, although experience of Bertrand's paradox³ warns that such an estimate may only be correct to an order of magnitude. Let the number density of quasars be n per unit area, and consider any one quasar. Between a distance x and $x + dx$ of this quasar will lie $2\pi nx \, dx$ other quasars. A line between the first quasar and any one of these others will be of length x , and any quasars which will form triplets with distance P or less away from this line must lie within a rectangular area $2xP$, and the number of such quasars will be $2xnP$. Thus the number of alignments with the original quasar at one end is

$$\int_0^d 4\pi x^2 n^2 P \, dx = \frac{4\pi}{3} d^3 n^2 P$$

where d is the largest outer quasar separation we consider. For a plate of area A , the total number of quasars is nA , so neglecting edge effects and with a factor of $\frac{1}{2}$ to avoid counting each alignment twice, the total number of alignments N where the third quasar lies within a distance P of the line joining the other two is

$$N = \frac{2\pi}{3} A d^3 n^3 P$$

Table 2 The distribution of d (the separation of the outer two quasars of any alignment) for which $P < 6.0$ arc s

d (arc min)	Mean of 16 runs	Largest no. in a single run	Smallest no. in a single run
0–10	0.4	2	0
10–20	1.3	3	0
20–30	3.9	7	2
30–40	7.6	13	3
40–50	11.5	17	5
50–60	17.3	27	12

For comparison with the computer simulation we note $N \propto P$ and $N \propto d^3$ (as found); and if $A = 50$ square degrees, $n = 6$ per square degree, $P = 2.4$ arc s $= 6.7^\circ \times 10^{-4}$, $d = 1^\circ$ we gain $N = 15$, in excellent agreement with Table 1.

We have not considered the redshifts of the quasars. To do so would require detailed knowledge of the redshift frequency distribution, which is not well determined. Also, the cosmological interpretation of the quasar phenomenon allows for the redshift distribution to be quite independent of the distribution of quasars in the sky. Hence we could superimpose the redshifts on the existing data once the redshift distribution is sufficiently well known. Of course, one might expect one-third of the alignments to have the central quasar of lower redshift than the two outer ones. Also important may be observational selection of a central quasar on the basis of a bright magnitude, which would affect comparison with alignment probability estimates. However, for the present purposes we neglect magnitude effects (which are difficult to quantify and will be correlated with the redshift distribution).

Our computed results give an indication of how many good alignments might be expected on 50 square degrees of sky, and hence we would expect 11 alignments good to 2.4 arc s and of overall size less than a degree to appear on a typical Schmidt

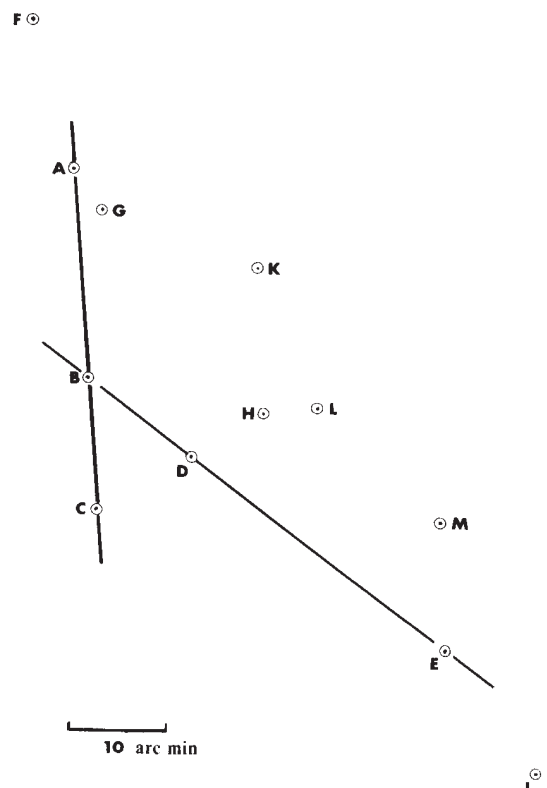


Fig. 1 An interesting square of sky (from random distribution of quasars). A, B and C are aligned to less than 0.2 arc s and B, D and E to 1.0 arc s. Also note that F, G, D and H, E, J form very good alignments (to within 2.0 arc s).

plate. It is debatable whether these are too loose criteria, and comparison with Arp and Hazard's configuration depends critically on the assumed values of n , d and P . It is particularly difficult to quantify d , the overall separation of triplets searched for. We therefore also consider a more restrictive case, and take the largest alignment found as d . Their four quoted alignments have $d < 30$ arc min, and should it be found that $P \leq 1$ arc s, then for $n = 6$ per degree we estimate an expected number of alignments $N = 0.5$ per Schmidt plate, somewhat relaxed to $N = 2.5$ if $n = 10$. Arp and Hazard's find of four alignments (albeit on an incomplete search) lies between our loose and restrictive estimates and is anyway not too great a fluctuation difference from the restrictive one. We suggest that the evidence is not strong enough to require a ballistic theory to explain their observed configuration, it could well have arisen by chance. *A posteriori* statistics on a single configuration is never satisfactory, and a more definitive test might be made by identifying all probable quasars on the plate and testing the properties of their mutual alignments against the statistics for random distribution developed above.

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Precursor active galaxies and the cosmic X-ray background

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Data on the isotropic extragalactic X-ray background (>3 keV) obtained with the HEAO 1 A2 instrument¹ yield a spectrum over the energy band of maximum flux that is well described by a thermal model involving an optically thin hot plasma at a temperature of a half-billion degrees ($kT = 40 \pm 5$ keV). The spectra for known strong X-ray sources in the present epoch (active galaxies, clusters of galaxies) obtained with the same HEAO 1 instrument^{2,3} indicate that such objects at high redshifts cannot account for most of this background. On the other hand, deep-field exposures to soft X rays (<3 keV) with the HEAO 2 Einstein Observatory⁴ suggest that discrete sources could be a sufficient explanation. This implies either (1) that these dim sources are generally unrelated to relatively bright nearby objects or (2) that they represent an earlier stage in their evolution. Short-lived thermal X-ray emission by galactic winds peculiar to an earlier epoch has been suggested⁵ as an explanation of the first kind, but the need for postulating new sources can be obviated if, as Leiter has argued⁶, prolific X-ray sources evolve in connection with active galactic nuclei involving massive compact objects. Here, we pursue the suggestion by Carr⁷ that most of the cosmic X-ray background (CXB) arises from thermal X-ray emission associated with black hole accretion disks for compact objects formed before galaxies but we favour a model where the onset of significant X-rays takes place within the epoch of galaxy formation at $z \leq 4$, when accretion disks are created by the interaction between massive black holes of pregalactic origin and young galaxies.

The spectral characteristics of the composite X-ray flux associated with the extragalactic sky is shown in Fig. 1. An extrapolation of power law (B) to the γ -ray regime (>1 MeV) suggests that such sources could well account for most of the

observed cosmic γ -ray background^{8,9}. But, as shown in Fig. 1, they can account for a relatively small part of the CXB.

For a survey limit of 2.6×10^{-14} erg cm⁻² s⁻¹ (1–3 keV), the deep exposures with HEAO 2 Einstein Observatory⁴ yield 19 ± 8 extragalactic sources deg⁻², comparable with the number of quasars¹⁰ brighter than $B \approx 20$ mag. Assuming that these sources have power-law X-ray spectra of energy index $\alpha = 0.7$ and that the slope a of the log N –log S relation at the survey limit and above is greater than unity, then these observed sources account for a percentage of the CXB which, at 3 keV, is given by $(a/(a-1)) (7.1 \pm 2.9)\%$. In Fig. 1, $a = 1.5$ (see ref. 4). A subsequent direct determination¹¹ of the log N –log S relation for these dim sources with HEAO 2, however, indicates that the slope is steeper and could be closer to $a = 2$. Yet, the log N –log S relation for quasars must flatten¹² as the number brighter than $B = 22.5$ mag is ≤ 50 deg⁻². The assumption that most of the CXB arises from quasars apparently gives a paradox for the required source count. Extrapolating the HEAO 2 source count through a log N –log S relation of constant slope a , for objects dimmer than the survey limit, we obtain that the number of sources required to account fully for the background is $\sim 2 \times 10^3$ sources deg⁻² for $a = 1.5$ and $\sim 10^3$ sources deg⁻² for $a = 2$. These estimates are an order of magnitude larger than the number of usual quasars considered which suggests that the onset of thermal X-rays precedes the formation of non-thermal radiating components.

The thermal-type spectrum observed for the CXB can be associated with two kinds of black-hole accretion disk radiation mechanisms: (1) bremsstrahlung from an optically thin hot plasma of the disk^{7,13}; (2) unsaturated Comptonization¹⁴ of soft photons by hot thermal electrons in the inner region of the disk, optically thin to absorption, where e^-e^+ pair production cooling effects¹⁵ can limit the electron temperature (T_e) to $T_e \approx 2 \times 10^9$ K for a source luminosity close to the Eddington limit. The consistency of our model requires that the main sources of the CXB radiate at a level close to the Eddington luminosity limit. As case (1) is constrained to luminosities $\leq 1\%$ of this limit¹³, we concentrate on case (2). For reasonable sizes of the emitting hot optically thin inner disk region¹⁴ ($\leq 10^2$ GM/ c^2), e^+e^- cooling¹⁵ provides a thermostat whereby $kT \approx 0.2$ MeV, implying that $z \leq 4$ for most such sources of the CXB. The same condition applies to case (1) as the Gaunt factor for optically thin thermal bremsstrahlung emission at $kT \approx 0.2$ MeV would fail to provide an acceptable fit to the CXB spectrum observed (R. Shafer, personal communication). This suggests that there is no accretion disk during a black-hole accretion phase earlier than that corresponding to $z \approx 4$. For photon energies much less than the observed redshifted value of $kT \sim 40$ keV the CXB exhibits an effective energy spectral index^{1,2} $\alpha \approx 0.4$. In this restricted regime, the CXB spectrum may be synthesized by a superposition of sources having power-law spectra with $\alpha \approx 0.4 \pm 0.1$ (G. DeZotti, personal communication). For a source temperature fivefold higher than that observed for the CXB (that is, $(1+z) 40$ keV = 200 keV), case (2) provides acceptable spectral indices for values of the Kompaneets parameter ($y \approx 1-2$)

Table 1 PAG sources of the CXB

Central mass $M_0 \leq 1$ ($M_0 \equiv 10^{-9} M/M_\odot$)
Black-hole specific angular momentum $0 \leq a/M \leq 0.998$ ($a = J/M$)
Luminosity (10^{47} erg s ⁻¹) (0.4–1.3) M_0
Number (4–8) $\times 10^7$
Thermal characteristics
Temperature: 2×10^9 K
Size: $\leq 10^2$ GM/ c^2 = $(2 \times 10^{16}) M_0$ cm
Emission mechanism: unsaturated comptonization (with Kompaneets parameter $y = 1-2$)
Redshift $z = 4$; $\Delta z = 0.3 (q_0 = 0.01)$ or $\Delta z = 0.4 (q_0 = 0.5)$
Composite luminosity (10^{54} erg s ⁻¹) [*] = 1.4 ($q_0 = 0.01$) or 0.3 ($q_0 = 0.5$)
Co-moving density (10^{-4} Mpc ⁻³) [*] = 1 ($q_0 = 0.01$) or 7 ($q_0 = 0.5$)

* For $h = H_0/(100 \text{ kms}^{-1} \text{ Mpc}^{-1}) = 0.5$ where H_0 = Hubble constant and q_0 = deceleration parameter.

characteristic of the model¹⁴. For photon energies $E \approx kT$, all these power-law spectra are modified by the required exponential tail-off: $\exp(-E/kT)$.

We start with Carr's suggestion that black holes are generated and evolve towards masses up to $\sim 10^9 M_\odot$ by pregalactic processes beginning at $z > 100$. However, the thermostat associated with the temperature observed for the CXB would imply that these black holes develop significant accretion disks at $z \lesssim 4$. Before the formation of these disks, random pregalactic processes which involve spherical accretion are not expected to be efficient for X-radiation¹⁶. Therefore, the X-radiation associated with accretion must be relatively unimportant until galaxy formation processes supply the matter associated with an ample accretion disk. Furthermore, pregalactic processes associated with random accretion should not cause appreciable spin-up of the black holes because the capture cross-section for negative angular momentum particles (opposite to the hole's spin) is greater than that for positive angular momentum¹⁷. Beginning at $z \lesssim 4$, within the epoch of galaxy formation, these massive black holes acquire significant accretion disks for the first time and become the 'seeds' which distinguish precursor active galaxies (PAG) from galaxies which will evolve normally. These PAG sources of the CXB postulated here are characterized in Table 1. In this model, these PAG objects would be the thermal sources of the sort required for the CXB, but necessarily cooler than previously considered ($kT \lesssim 0.2$ MeV). In the process of disk accretion on a time scale of $\sim 10^8$ yr, however, the central massive black hole of a PAG could increase in mass by a factor of ~ 1.5 and be spun-up¹⁸ to a 'canonical Kerr hole'. By this time black hole ergospherically induced²⁰ electron-positron injection^{6,19,33} could trigger and surge electromagnetic disk-dynamo mechanisms^{21,22}. Furthermore, the efficiency for energy transfer to the relativistic particles required for non-thermal radiation processes (such as synchrotron) could by then begin to increase appreciably, as the size of the acceleration region grows^{23,24}. Hence, during transition, there may be some young active galaxies which are not yet sufficiently luminous non-

thermal disk-dynamo synchrotron sources to be readily identified as such while their thermal disk X-ray emission is still large.

The spin-up of a black hole due to a radiating accretion disk reaches an equilibrium 'canonical' value¹⁸ ($a/M \approx 0.998$) when the hole's mass increases by $\sim 1.5 M$ (initial). Assuming that the radiation efficiency (ϵ) is constant and that the luminosity (L) is proportional to the Eddington limit (L_{Edd}), this increase of mass is achieved in a time (Δt) after the formation of the accretion disk, given by

$$(\Delta t) = 4 \times 10^8 \epsilon (1 - \epsilon)^{-1} R^{-1} \text{ yr} \quad (1)$$

where $R \equiv L/L_{\text{Edd}}$. In such conditions the black hole mass and size increase exponentially with a characteristic growth time given by equation (1). More realistically, ϵ changes during the accretion process. Initially, when $\epsilon \approx 0.06$, this growth time could be as short as 3×10^7 yr (for $R \approx 1$). At spin-up¹⁸, however, $\epsilon \approx 0.3$ and the characteristic time for further growth would be at least an order of magnitude longer (with $R < 1$). If the accretion rate is then 'starved' by a high angular momentum configuration of remaining gas, such as in a spiral galaxy, we might expect $R \ll 1$ and that any further growth within a Hubble time ($\sim 10^{10}$ yr) is relatively minor. The amorphous nature of the spiral galaxies associated with Seyfert nuclei may well be caused by a process which slowly feeds material from outer regions into the centre²⁵.

By the time of spin-up, given by equation (1), the innermost stable orbit of the accretion disk has amply penetrated the ergosphere²⁶, and electron-positron injection processes⁶ associated with a canonical Kerr black hole are 'switched on'. The time (Δt) to reach this state is the effective lifetime of the PAG phase. This canonical state of spin is insensitive to small ($< 10\%$) fluctuations in the matter accretion rate and the counter-torques due to radiation¹⁸. In this context, the evolution of a PAG into a system involving a massive canonical Kerr hole may be viewed as an essentially irreversible process. Such behaviour is of the type needed for describing the observed evolution²⁷ whereby the sources of the CXB (for example, PAG objects at $z \lesssim 4$) lead directly to the active galaxies observed at later epochs. As a broad band of non-thermal radiation can eventually be generated at a relatively high efficiency²⁸ (at the expense of weakened thermal disk emission^{21,22}), the thermal disk component could be masked for many of the brightest active galaxies, but might sometimes be observable. In this connection, the spectrum measured for the quasar 3C273 is consistent²⁹ with a substantial contribution from a thermal X-ray component at $T \gtrsim 10^9$ K, while the broad-band X-ray spectrum of QSO 0241 + 622 is incompatible with such a feature³⁰. The PAG objects associated with Seyfert nuclei might involve galaxies where the amount of gas available for accretion from a low angular momentum configuration (for example, in a central bulge) is not large compared with that of the initial black hole. In this situation (such as one involving a spiral galaxy) we expect that a luminosity as high as 10^{47} ergs s^{-1} (Eddington limit for $10^9 M_\odot$) could not last much beyond spin-up. For appreciably longer time scales we assume $R < 1$; the resulting active galaxies could thereby survive to be observed in the present epoch (for example, as Seyfert galaxies) with much lower luminosities but with non-thermal spectra extending well into the γ -ray band.

The thermal component of the CXB corresponds to a surface brightness of 2.0×10^{-7} erg $(\text{cm}^2 \text{ s sr})^{-1}$. For a Friedman cosmology with $q_0 \leq 0.5$, $H_0 = 100 h \text{ km s}^{-1} \text{ Mpc}^{-1}$ and with most sources of the CXB at $z \approx 4$, the bolometric luminosity for the sum of all these sources, in their proper frame, is $\Sigma L \gtrsim 8 \times 10^{52} h^{-2} \text{ erg s}^{-1}$. The number of sources required for the CXB is $N(\text{CXB}) \approx (4-8) \times 10^7$, so the implied average bolometric luminosity per source ($\bar{L} \gtrsim 10^{45} h^{-2} \text{ erg s}^{-1}$) is readily associated with the PAG objects postulated here as the limit $R > 0.3$ is required to constrain the PAG lifetime from being much greater than $\sim 10^8$ yr (see equation (1)). If the central black hole were initially much less massive than $\sim 10^7 M_\odot$, then it would take substantially more than 10^8 yr (beyond spin-up) to

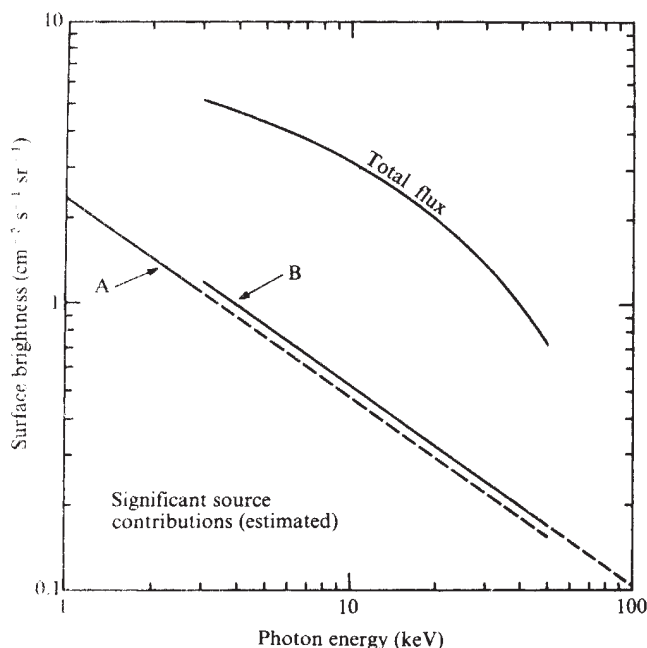


Fig. 1 Spectral density for the surface brightness of the extragalactic X-ray sky as a function of photon energy. The 'total flux' curve is the best-fit thermal spectrum ($kT = 40$ keV) for the background measured by Marshall *et al.*¹. The power-law 'A' represents the composite flux due to sources detected above the survey limit in the HEAO 2 deep exposures by Giacconi *et al.*⁴. The power-law 'B' represents the composite flux from active galaxies with $z \lesssim 1$, based on the local luminosity function determined³ with HEAO 1 A2 and assuming no evolution, with $q_0 = 0$. The dashed lines are extrapolations.

grow towards a mass where the luminosity could reach the level required here for sources at $z \approx 4$. Hence, such objects would not qualify as PAG sources of the CXB.

The total number of PAG objects $N(\text{PAG})$ making up the CXB is fixed by the model in terms of the local density of active galaxies. A fundamental aspect of this model is that the density of PAG objects in co-moving coordinates remains constant during the time (Δt) of spin-up and that the co-moving density of active galaxies after production equals that of the associated PAG objects. Assuming that the PAG population is produced at a look-back time corresponding to $z = 4$, $N(\text{PAG})$ is given by

$$N(\text{PAG}) = (1.4 - 8.9) \times 10^8 \phi \Delta\tau h^{-3} \quad (2)$$

where the limits indicated correspond to $q_0 = 0.5$ and $q_0 = 0.01$ respectively, $\Delta\tau$ (units of age of the universe) is the look-back temporal interval of the PAG phase, and ϕ is the co-moving density of PAG objects (in units of 10^{-4} Mpc^{-3} , the value measured²⁵ for the local density of Seyfert galaxies and consistent with the local luminosity function for X-ray active galaxies³ in the luminosity range $\sim 10^{42} - 10^{45} \text{ erg s}^{-1}$ (3–50 keV)). For $\phi \geq 1$ and $\Delta\tau \approx 10^{-2}$ (corresponding to $\sim 10^8 \text{ yr}$) equation (2) indicates that the number of PAG objects could well match the number of sources required for the CXB: $N(\text{CXB}) \approx (4-8) \times 10^7$. The total mass involved in all the pregalactic black holes that precipitated these sources would be less than 10^{-4} of the known mass in galaxies. When most PAG objects are within $\Delta\tau = 10^{-2}$ at $z \approx 4$ then $\Delta z < 8 \times 10^{-2}(1+z)$, ensuring that the fractional spread in observed temperatures due to redshift would be $< 8\%$, compatible with the CXB spectral data.

We conclude that most of the CXB could be generated by distant sources that are not usual active galaxies or quasars, and that they must have a relatively low ratio of optical to X-ray luminosity (that is dominantly X-ray objects). PAG objects at $z \leq 4$ involving a massive ($\leq 10^9 M_\odot$) black hole surrounded by a hot optically thin accretion disk radiating near the Eddington luminosity limit with an effective temperature $\sim 2 \times 10^9 \text{ K}$ would meet this condition and yield the correct CXB spectrum and intensity. Furthermore, this model has the advantage of providing an evolutionary track which links both the CXB and the non-thermal γ -ray background directly to active galaxies, implying that massive ($\leq 10^9 M_\odot$) black holes are indeed associated with their internal dynamics (as suggested by X-ray observations³¹ involving temporal variability). While the PAG sources of the CXB postulated here would have low optical continuum emission relative to quasars, strong emission lines comparable with those of quasars generated from a broad-line region photoionized³² by the central X-ray source could lead to a direct identification of these objects. As the average X-ray flux expected from a PAG is an order of magnitude below the survey limit for the HEAO 2 Einstein Observatory, they would not be well represented in the 'complete' sub-sample observed, but some of the dimmest unidentified objects detected should be considered candidates.

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Measurement of ionospheric winter anomaly in Southern Hemisphere

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The unexpectedly high ionospheric radio-wave absorption that occurs during winter at temperate latitudes is referred to as the 'winter anomaly'. These variations have been attributed to increases in the concentration of the minor atmospheric species NO and O₂(¹Δ_g), themselves important in the formation of the D-region. This anomaly involves an order of magnitude increase of the electron number density which, in turn, leads to an increase in the absorption experienced by radio waves propagating in the D-region. The phenomenon occurs between 75 and 95 km, with the maximum enhancement near 80–83 km (ref. 1). We describe here a period of anomalous absorption observed at Camden, Australia (34°S, 151°E), during 15–17 June 1980. This anomalous event is significant because previous observations of the winter anomaly have, in general, been restricted to the Northern Hemisphere, especially Europe and because our observation was made at a lower latitude than those suggested^{2,3} as the low-latitude boundary of the winter absorption anomaly. Previous measurements in the Northern Hemisphere have placed the low-latitude boundary at about 37°N (for the 1968–69 winter²) and 35°N (for the 1971–72 winter³). The second measurement suggested an increase of 1 dB per degree of latitude above the boundary. The magnitude of the anomalous absorption in our measurements, at 34°S, was 10 dB. The magnitude of this absorption suggests either that this event was very large or that the ionospheric response is different in the two hemispheres.

The A1 (or pulse amplitude) method for measuring ionospheric absorption involves the measurement of the amplitude of radio-wave pulses reflected vertically from the ionosphere. An automatic A1 system, which uses a micro-processor for both the various system control functions and the signal processing has been described elsewhere⁴. The equipment was installed at Camden, New South Wales, in December 1979 and operates on a frequency of 1.91 MHz. A sample of the amplitude data (3-min medians) for 20 June 1980 is plotted in Fig. 1 as a function of local time (GMT + 10 h) between 08.00 and 16.00 h.

A convenient technique for characterizing the diurnal variation in the amplitude data is to fit a second-degree polynomial (quadratic) to the observations by using least-square methods. For instance, the quadratic which best fits the observations for 20 June between 08.00 and 16.00 h is shown in Fig. 1. The scatter of points about this (solid) curve is attributed mainly to either non-dissipative amplitude fluctuations (fading) or to variations in the reflection mode. In addition, solar X-ray events can cause the data points to depart significantly (by $> 10 \text{ dB}$) from the quadratic curve describing the normal daily variation in the amplitude data. The ionospheric response to X rays of short

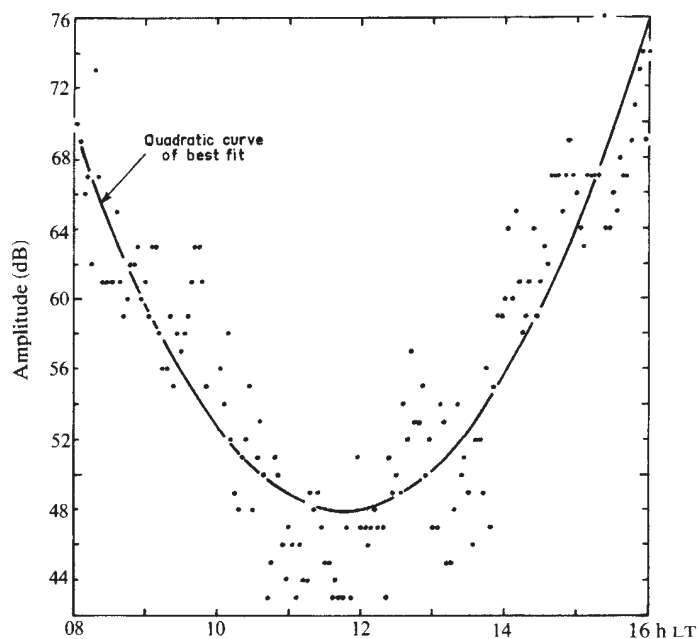


Fig. 1 A1 amplitude data (3-min medians) for the period 08.00–16.00 h, 20 June 1980. The full curve represents the second-order polynomial of best fit through the data points.

wavelengths ($<10 \text{ \AA}$) is to increase the concentration of electrons in the D-region through ionization of the neutral atmosphere. This is accompanied by an increase in the absorption suffered by radio waves as they travel through the region of enhanced electron density.

The quadratic curves of best fit (one for each day) for the periods 8–20 June (solid curves) and 4–11 July (broken curves) are superimposed in Fig. 2. A1 data for the intervening period were not available. Solar X-ray events during these periods were either of small intensity (C class events) or of small duration ($<1 \text{ h}$), which indicates that the ionospheric response was not large enough to modify significantly the coefficients of the quadratic curves of best fit. However, this was not true for the data collected on 6 July, during which a large (M9) X-ray event produced an absorption increase of at least 60 dB between 08.40 and 09.05 LT. Consequently, the A1 amplitude data associated with this event were omitted in determining the quadratic curve for 6 July.

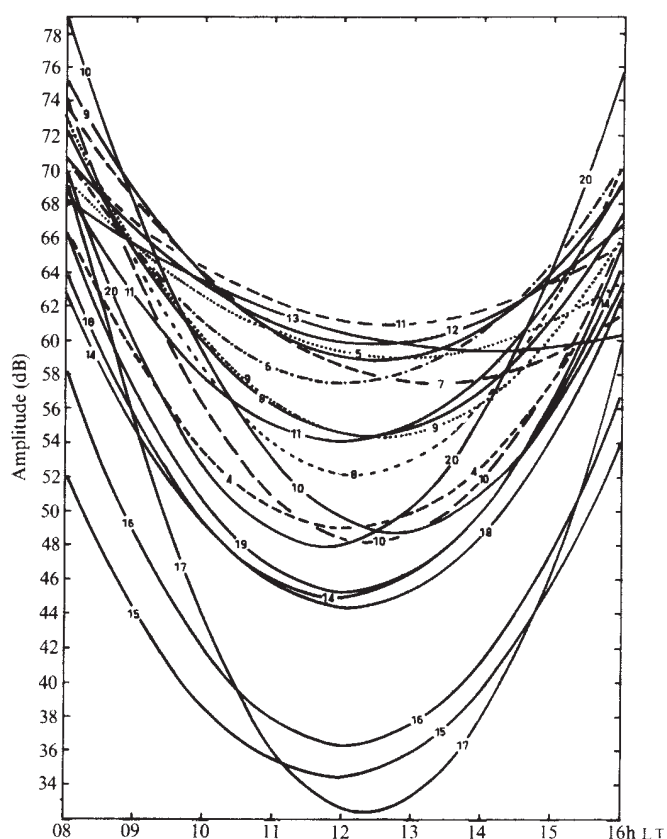


Fig. 2 Quadratic curves of best fit for the A1 amplitude data for the periods 8–20 June (solid curves) and 4–11 July (broken curves). The days that exhibit marked anomalous absorption are 15, 16 and 17 June 1980. Numbers on the curve indicate days of the month.

Figure 2 shows that the days of 15–17 June were characterized by amplitude values which were consistently below those for the other days. Thus 15–17 June are classified as days which exhibited anomalous absorption. The minimum amplitude values of the quadratic curves associated with these anomalous days are $\sim 10 \text{ dB}$ less than those for the adjacent days of 14, 18 and 19 June. However, by considering days remote from the anomalous period (say 10 June and 10 July), the anomalous absorption seems to be $\sim 15 \text{ dB}$. If the latter estimate of the magnitude of

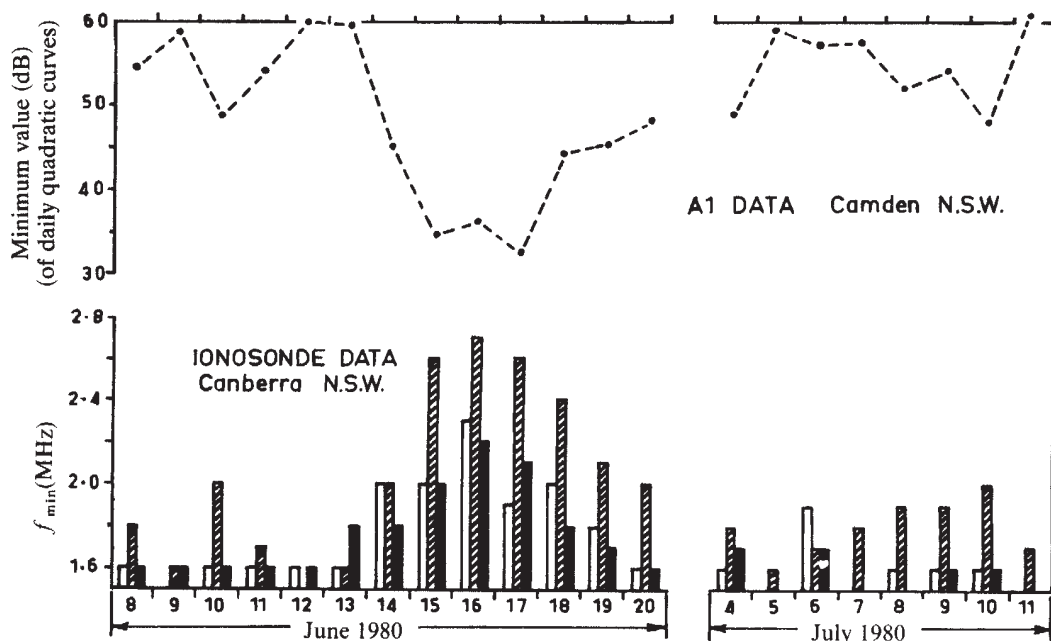


Fig. 3 Minimum values of the daily quadratic curves of best fit for the Camden A1 data, together with a histogram of the Canberra $f_{\min}$ data (median of the 3-h values), plotted for the periods 8–20 June and 4–11 July 1980. $\square$, 08.00–10.00 h; $\square$, 11.00–13.00 h; $\blacksquare$, 14.00–16.00 h LT.

the anomalous absorption is adopted, then the period associated with the winter anomaly can be extended to include 14, 18 and 19 June. Thus the anomalous absorption for 14, 18 and 19 June was ~3 dB, while for 15–17 June it was ~15 dB.

The winter anomaly in absorption also manifests itself in the Canberra (35°S, 149°E) ionosonde data. The appropriate parameter is $f_{\min}$ which is the lowest frequency at which an echo returned from the ionosphere can be detected on an ionogram. This parameter is observed to increase as absorption increases, for example, during solar X-ray flares. However, $f_{\min}$ is also affected by factors extraneous to ionospheric absorption, for instance, equipment characteristics and radio-frequency interference. Figure 3 shows the medians of the scaled $f_{\min}$ hourly values for the periods 08.00–10.00, 11.00–13.00 and 14.00–16.00 h using a histogram format for the periods 8–20 June and 4–11 July. For comparison, a graph of the minimum values of the quadratic curves of best fit for the A1 data is also shown for the same periods. The days characterized by enhanced $f_{\min}$ values coincide with days for which the absorption was anomalously high.

The above preliminary analysis indicates that a period of anomalous absorption occurred during the winter of 1980 in the Southern Hemisphere. The A1 amplitude data from Camden indicated that the anomalous absorption had a lower limit of 10 dB and may have been as high as 15 dB, depending on the reference level. Enhanced $f_{\min}$ values were recorded on the anomalous days by an ionosonde located in Canberra. The results show that an asymmetry exists between the Northern and Southern Hemispheres with respect to winter anomalous absorption. Predictions based on data from only the Northern Hemisphere were found to be inappropriate as they failed to explain the existence of anomalous absorption at Camden. This suggests that the experimental programmes aimed at collecting data on the aeronomic (physical, chemical and dynamical) behaviour of the stratosphere/mesosphere during the forthcoming Middle Atmosphere Programme (1982–85), should be conducted in both hemispheres. The results should be analysed separately (according to hemisphere) rather than combining them into one data base which would average-out any asymmetry. Also, global aeronomic models should reflect the differences that exist between the hemispheres.

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Inorganic lead complexation in natural seawater determined by UV spectroscopy

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Recent effort to construct trace metal complexation models in natural media^{1–8} is a mark of the importance of chemical form in the biogeochemical behaviour of trace metals. Unfortunately such chemical modelling is difficult in many ways. Chemical speciation models of trace metals in complex natural media such as seawater are usually constructed using stability constants determined in simple media. We describe an alternative procedure which can reduce the errors inherent in this approach. Using UV spectroscopy, we have examined lead speciation in seawater. The method is a means of describing metal speciation in seawater quantitatively using stability constants determined exclusively in seawater. Preliminary experiments indicate that the same approach can be used for other metals including copper.

Speciation models for lead in seawater (Table 1) alternatively predict: (1) that only lead carbonate complexes¹ are important; (2) that lead carbonate species are unimportant and that lead chloride complexes are dominant^{2,3}; (3) that lead hydroxide complexes are important^{4,5}; and (4) that lead hydroxide complexes are unimportant⁶. Sipos *et al.*⁵ have described two models of lead complexation in seawater. In their second model, they adopt the chloride speciation scheme of Johnson and Pytkowicz⁹ and the concentration ratio of their dominant lead species, $\text{PbOH}^+/\text{PbCO}_3^0$, diminished by a factor of 3.5 compared with the first model. Three factors explain the observed variability of models. (1) Stability constants are selected from a wide range of values. (2) Stability constants are generally determined in simple media and adapting these constants to seawater conditions introduces error. (3) Minor element speciation schemes depend on the speciation schemes adopted for the major constituents of seawater. Our approach eliminates the latter two sources of error, and provides an essential test of the various models constructed for lead speciation in seawater.

Lead chloride complexation experiments used unfiltered surface seawater (34.7‰ salinity) from the Gulf of Mexico which was enriched in lead (~7 μM in Pb(II)) with an acidic lead stock solution. The UV absorbance characteristics of these solutions were measured between 300 and 220 nm against a lead-free seawater reference solution. The test solution, housed in a 10-cm pathlength spectrophotometric cell, was acidified with varying amounts of 1 M HCl, producing a series of absorbance versus pH curves at 25 °C (Fig. 1). Cells were sealed except during measurements of pH. For each absorbance versus wavelength curve (Fig. 1) the solution pH ($-\log A_{\text{H}}$) was determined with a micro combination pH probe (Micro-electrodes Inc.) calibrated on the NBS scale. Experiments were conducted at the naturally occurring total CO_2 concentration, 2.05×10^{-3} mol total CO_2 per kg seawater. Total CO_2 and M_{CO_3} , the total carbonate ion concentration, were calculated from pH and the initial carbonate alkalinity^{10,11} using the apparent constants of Mehrbach *et al.*¹².

The isosbestic point observed at 237 nm in Fig. 1 indicates that adsorption is not significant. The strong pH dependence demonstrates that lead is not exclusively complexed with chloride ions in seawater. PbCO_3^0 and PbOH^+ are thought to be important at high pH. Varying the pH of a lead-enriched seawater solution between 3.9 and 8.2 causes molar-absorptivity changes between 220 and 300 nm which are highly dependent on the seawater's total CO_2 content. The molar-absorptivity change is $<250 \text{ M}^{-1} \text{ cm}^{-1}$ at zero total CO_2 and as large as $1,400 \text{ M}^{-1} \text{ cm}^{-1}$ at the natural CO_2 concentration, 2.05×10^{-3} mol per kg seawater. The much larger change when carbonate is present suggests that PbOH^+ is much less significant than PbCO_3^0 . Hence plots of absorbance versus pH at constant wavelength will be distinctly different for different total CO_2 concentrations, and absorbance versus $\log M_{\text{CO}_3}$ at different pH will be nearly identical (Fig. 2).

The results of five experiments were analysed quantitatively: three at total CO_2 2.05×10^{-3} mol per kg seawater and two at total CO_2 9.84×10^{-3} mol per kg seawater. Assuming PbOH^+ is insignificant compared with PbCO_3^0 , the absorbance of lead in seawater can be described by^{13,14}:

$$_{\lambda}A = \frac{\epsilon_0 + \epsilon_1 \beta_1 M_{\text{CO}_3}}{1 + \beta_1 M_{\text{CO}_3}} \quad (1)$$

where $_{\lambda}A$ is absorbance at wavelength λ , M_{CO_3} is the sum concentration of carbonate species (such as, CO_3^{2-} , NaCO_3 , MgCO_3^0 , and CaCO_3^0), ϵ_1 is absorbance due to PbCO_3^0 , ϵ_0 is absorbance due to the lead forms which dominate at low pH (principally Pb^{2+} , PbCl^+ , PbCl_2^0 and PbCl_3^-) and β_1 is the formation constant of PbCO_3^0 .

Lead-absorbance data were analysed at four wavelengths (240, 250, 260 and 270 nm) which did not require corrections for baseline variability with pH. Absorbance data at these

Table 1 Species composition of inorganic lead in seawater

Model species	Ref. 2*	Ref. 3	Ref. 7	Ref. 4	Ref. 5†	Ref. 8	Ref. 6	Ref. 1	
PbCO ₃ ⁰ , Pb(CO ₃ ²⁻) ₂ ²⁻ , PbCO ₃ Cl ⁻	—	<1	18	40	22	47	65	77	100
PbOH ⁺ , Pb(OH) ₂ ⁰ , PbOHCl ⁰	2	15	5	33	49	30	6	1	—
Pb ²⁺ , PbCl ⁺ , PbCl ₂ ⁰ , PbCl ₃ ⁻ , PbSO ₄ ⁰	98	84	73	27	28	23	28	21	—

Composition is given as a percentage of the total inorganic lead at pH 8. Totals <100% indicate that additional complexes contribute to the speciation scheme.

* The Morel and Morgan² model was constructed for a hypothetical system which is quite similar to seawater in ionic strength and the free concentrations of Cl^- , CO_3^{2-} and H^+ .

† The Sipos *et al.*⁵ model is constructed for pH 8.1.

wavelengths in five experiments were fit using equation (1) in a non-linear least-squares analysis^{13,14}. At 25.0 °C and 34.7% salinity:

$$\beta_1 = \frac{M_{\text{PbCO}_3}}{M_{\text{Pb}} \cdot M_{\text{CO}_3}} = (10.0 \pm 0.3) \times 10^3 \quad (\text{mol per kg seawater})^{-1} \quad (2)$$

where M_{PbCO_3} is the concentration of PbCO_3^0 and mixed ligand carbonate species^{8,15,16}, and M_{Pb} is the sum concentration of Pb^{2+} , PbCl^+ , PbCl_2^0 , PbCl_3^- and less significant species. This PbCO_3^0 formation constant enables $M_{\text{PbCO}_3}/\text{total Pb}$ at 25.0 °C

contains a greater total CO_2 concentration, PbCO_3^0 seems to be the dominant form of inorganic lead in seawater.

Expansion of equation (1) to account for the possible existence of PbOH^+ , PbHCO_3^+ and $\text{Pb}(\text{CO}_3)_2^{2-}$ in the test medium produced β_1 values similar to that in equation (2). Although formation constants for the species PbOH^+ , PbHCO_3^+ and $\text{Pb}(\text{CO}_3)_2^{2-}$ were ill-defined suggesting that these species were present only in low concentration, the analyses did produce upper 95% confidence limits which are useful for estimating the importance of each species in seawater (Table 2). The formation constant determined for PbHCO_3^+ indicates that this species has little significance ($M_{\text{HCO}_3} = 2 \times 10^{-3}$ mol per kg seawater). From its formation constant, PbOH^+ is unlikely to constitute more than 10% of the total inorganic Pb in seawater ($\text{pH} \leq 8.3$). From the β_2 upper 95% confidence limit, $\text{Pb}(\text{CO}_3)_2^{2-}$ and PbCO_3^0 could be present at approximately equal concentrations in seawater ($M_{\text{CO}_3} \approx 3 \times 10^{-4}$ mol per kg seawater), but the existence of well defined isosbestic points makes this improbable. If $\text{Pb}(\text{CO}_3)_2^{2-}$ were present at appreciable concentrations, its molar absorbance at 237 nm would have to be identical to that of PbCO_3^0 to produce the observed isosbestic point.

The present analysis requires that a 7 μM lead enrichment would not significantly alter the inorganic complexation of lead in seawater. This is satisfied because the appropriate ligands have concentrations $\gg 7 \mu\text{M}$. The most unfavourable molar ratio, total $\text{CO}_2/\text{Pb(II)}$, is >290 . Consequently, calculated values of M_{CO_3} are more affected by small experimental errors in measurement of pH than by lead enrichment, enabling this description of inorganic lead complexation to be applied to natural seawater.

Our lead carbonate formation constants are strongly influenced by the ion pairing interactions of carbonate ion and possibly chloride ion with the major cationic constituents of seawater. However, as our methods do not require any particular model of these interactions, our formation constants and conclusions are independent of these descriptive models.

Constants of the form defined by equation (2) allow the pH dependence of Pb speciation in seawater to be described without assuming that activity coefficients are constant in diverse media at constant ionic strength. Our conclusions that the dominant

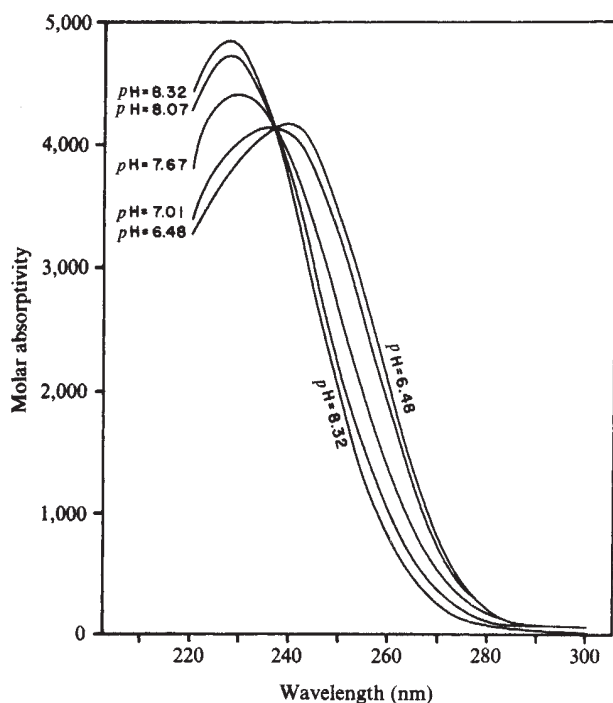


Fig. 1 Absorbance per molar cm of Pb(II) in natural seawater as a function of wavelength and pH. (Temperature 25.0 °C; salinity 34.7%; total CO_2 : 2.05×10^{-3} mol per kg seawater.) Absorbance data are corrected for baseline variation with pH at wavelengths <235 nm.

and 34.7% salinity to be calculated when total CO_2 and pH are known. M_{CO_3} is calculated from total CO_2 and pH (see refs 11, 12). The fraction of total lead present as PbCO_3^0 can then be determined without knowledge of the ion pairing interactions of CO_3^{2-} with the major cationic seawater constituents, and there is no formation constant variation between seawater and the test medium.

Using equation (2) at 25.0 °C, 34.7% salinity and total CO_2 of 2.05×10^{-3} mol per kg seawater it can be shown that $M_{\text{PbCO}_3}/\text{Total Pb} \geq 0.5$ for $\text{pH} \geq 7.85$. As seawater generally

Table 2 Results of formation constant determinations in natural seawater at 25.0 °C and 34.7% salinity

Species considered	Formation constants determined
PbCO_3^0	$\beta_1 = 10.0 \times 10^3$
PbCO_3^0 , PbHCO_3^+	$\beta_1 = 9.4 \times 10^3$; $\text{HCO}_3\beta = \frac{M_{\text{PbHCO}_3}}{M_{\text{Pb}}M_{\text{HCO}_3}} \leq 14.5$
PbCO_3^0 , PbOH^+	$\beta_1 = 10.3 \times 10^3$; $\beta_1 = \frac{M_{\text{PbOH}}A_{\text{H}}}{M_{\text{Pb}}} \leq 2 \times 10^{-9}$
PbCO_3^0 , $\text{Pb}(\text{CO}_3)_2^{2-}$	$\beta_1 = 12.3 \times 10^3$; $\beta_2 = \frac{M_{\text{Pb}(\text{CO}_3)_2}}{M_{\text{Pb}}M_{\text{CO}_3}^2} \leq 7.3 \times 10^7$

Concentrations of all chemical species except H^+ are defined on a mol per kg of seawater concentration scale. Hydrogen ion activities, A_{H} , are operationally defined on the NBS scale¹¹.

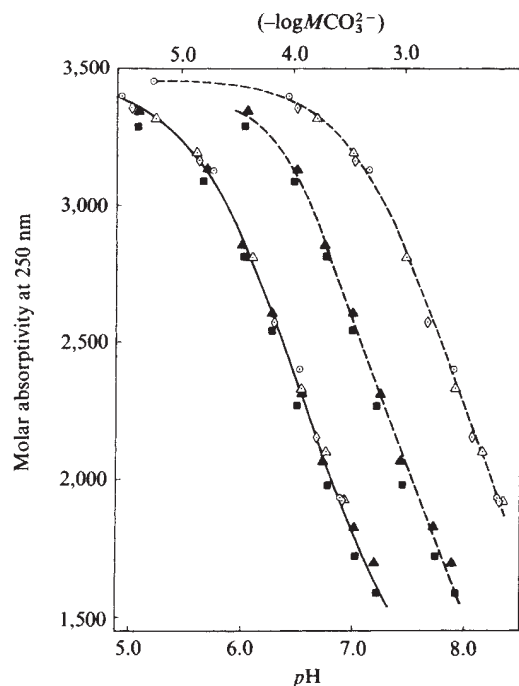


Fig. 2 Absorbance per molar cm of Pb(II) in seawater as a function of pH and log M_{CO_3} at 25.0 °C and 34.7% salinity. Total CO_2 is 2.05×10^{-3} mol per kg seawater (Δ and $\diamond$) or 9.84×10^{-3} mol per kg seawater ($\blacktriangle$ and $\blacksquare$). Dashed lines are coordinated on the pH axis. The solid line is coordinated on the log M_{CO_3} axis.

inorganic species of lead in seawater are $PbCO_3^0$ and lead chloride species are consistent with the models of Turner and Whitfield⁸ and Zirino and Yamamoto⁶.

Other trace metals such as iron (III) and copper have intense absorbance spectra in the UV. The methods used here can be extended to investigations of the chemistry of iron and copper in natural media. Preliminary experiments indicate that studies of copper in seawater can be conducted within the natural pH range of seawater. The plasticity of the copper (II) coordination sphere¹⁷ suggests that the activity coefficients of copper species may be significantly influenced by medium composition, and hence that the 'natural medium approach' may be especially useful.

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The age of the Weddell Basin

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The deep oceanic basin occupied by the Weddell Sea separates the East Antarctic craton from the Antarctic Peninsula, a largely post-Palaeozoic orogenic belt. An understanding of the tectonic evolution of the basin would resolve most of the remaining problems of Gondwanaland reconstruction, which revolve around the relative positions of these two fragments and South America. Norton and Sclater¹ conclude that Indian Ocean marine geophysical data essentially support the Smith and Hallam fit², but the unacceptable overlap of undoubtedly old continental crust^{3–5} on the Antarctic Peninsula and Falkland Plateau implies relative motion between the Peninsula and East Antarctica. Such motion is not required by the reconstruction of Barron and others^{7,8} which, however, can be criticized on other grounds^{9,10}. The marine geophysical observations within the intervening ocean basins have proved useful in formulating plate tectonic reconstructions. Fracture zone trends, seafloor spreading type magnetic anomalies, crustal heat flux, and crustal depth all provide clues to the timing and direction of the continental motions. The acquisition of geophysical data from the Weddell Sea region has been slow^{11–13}. However, the combined interpretation of the two largest data sets extant^{12,13} now being undertaken, seems likely to provide a more tightly constrained Gondwanaland solution. As a first step, we report here an estimated age for the Weddell Sea floor in the area bounded by 60° and 73° S, 5° and 35° W shown in Fig. 1.

Figure 1 also shows the tracks of cruises 1277 and 1578 of ARA Islas Orcadas in the Weddell Basin. Line drawings of seismic reflection profiles obtained on three long north–south lines during these cruises form Fig. 2. A zone W at 64°–65° S¹² separates a more northern area of much greater basement relief from the more southern area being considered here. In this northern area, fracture zone orientations are highly oblique to the gross orientation of the magnetic lineations^{12,13} but the more subdued basement topography to the south suggests that fracture zones there are subdued and have only small offsets. The general depth of the basement increases southwards and, when corrected for sediment loading, is consistent with a Mesozoic age^{12–14} in the area south of zone W with younger crust to the north.

Figure 3 shows our age model for the oceanic crust which floors the Weddell basin. The magnetic anomaly profiles plotted in Fig. 3 are from ref. 13 and are augmented by data collected aboard ARA Islas Orcadas and Project Magnet Flight 720. Note that the magnetic anomalies display a strong east–west lineation and seem to converge to the west. They are compared in Fig. 3 with a magnetic model covering the time period 162–35 Myr. (anomalies M29–13^{15–17}). Seafloor spreading rates vary from 0.6 cm yr^{–1} during the early Cretaceous to 0.9 cm yr^{–1} during the Palaeogene. To model the smoothing effect of finite polarity transition zones within the oceanic crust we have convolved the magnetic polarity model with a gaussian function ($\sigma = 3$ km)¹⁸. Rather than assume a magnetic pole position for the anomaly sequence, we have assigned a value of zero skewness to the pattern¹⁹. This effectively defines a family of probable pole positions though we must also account for anomalous skewness²⁰. The choice of zero skewness is based largely on the apparent symmetric nature of anomalies 33–34. All other anomalies can accommodate variations of up to $\pm 30^\circ$ in the model skewness.

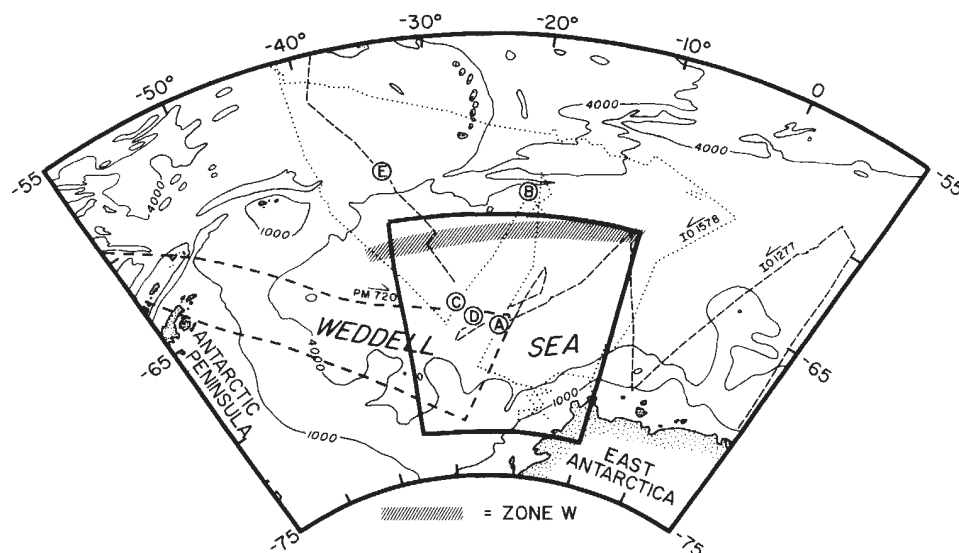


Fig. 1 Map of Weddell Basin showing regional location of Fig. 3. Tracks of Islas Orcadas cruises 1578 and 1277 are shown superimposed upon the regional bathymetry in meters²⁹. Encircled letters illustrate locations of seismic reflection profiles of Fig. 2.

Figure 3 also shows synthetic fracture zones or flow lines (stippled lines) calculated for the various reconstructions of Norton and Sclater¹. The flow lines display the displacement of a point on the Antarctica–South America spreading centre with respect to Antarctica fixed²¹. The flow lines are small circles about stage poles calculated for South America motion with respect to Antarctica fixed. We have included flow lines for an M13 reconstruction assuming that the change in plate motions required by the initiation of seafloor spreading in the South Atlantic^{22,23} was accommodated by a change in Antarctica–South America relative motions.

The spreading rates and directions inferred from the calculated flow lines match the observed data reasonably well with two exceptions. The flow line which represents crustal generation during the interval M1–34 (Cretaceous Normal) was reduced in length by 80% from that predicted by the Norton and Sclater poles. This suggests either a significant error in the

Norton and Sclater reconstructions for the time period, or an asymmetry in the seafloor spreading process such as a southward jump of the spreading centre. The dramatic change in spreading direction required by the opening of the South Atlantic in the Valanginian is not observed in the magnetic lineation pattern though a gradual reorientation of the anomaly pattern is perceived.

The strong lineation shown by anomalies 33–34 is consistent in direction and spreading rate with that predicted for Antarctica–South America motion at the Agulhas Magnetic Bight²⁴. The Weddell anomaly 33–34 sequence is a westward extension of the same anomaly sequence observed by Bergh and Barrett²⁵ north-west of the Maud Rise. The calculated lineation direction of the Bergh and Barrett sequence is in error, however, because of their assumption of symmetric seafloor spreading within the Agulhas Basin at the Africa–South America (or Malvinas) spreading centre²⁴.

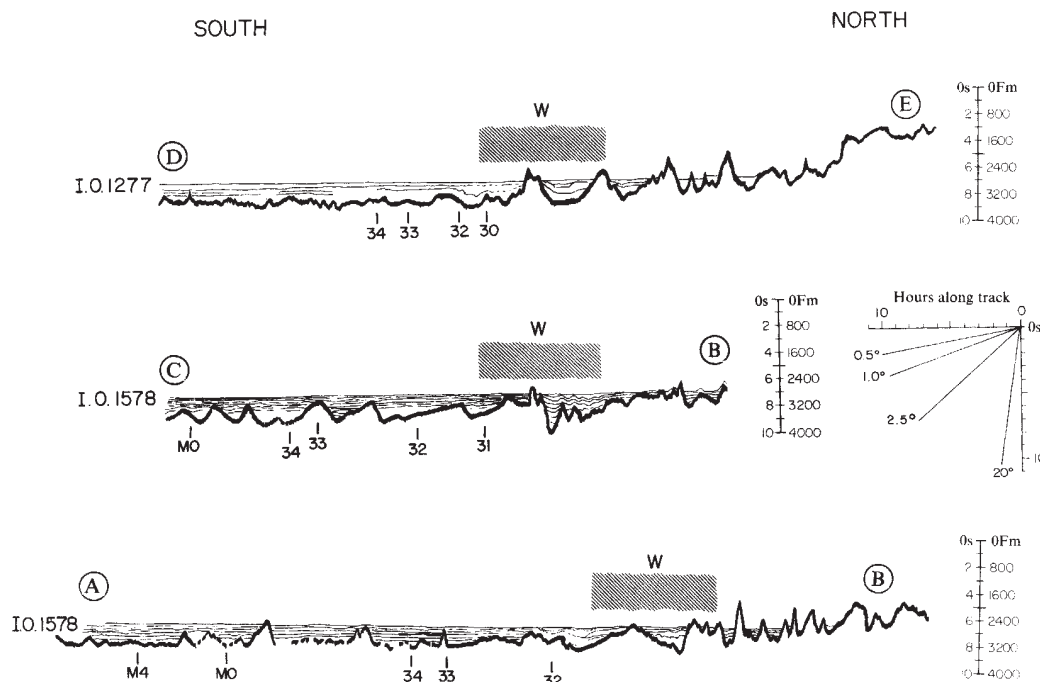
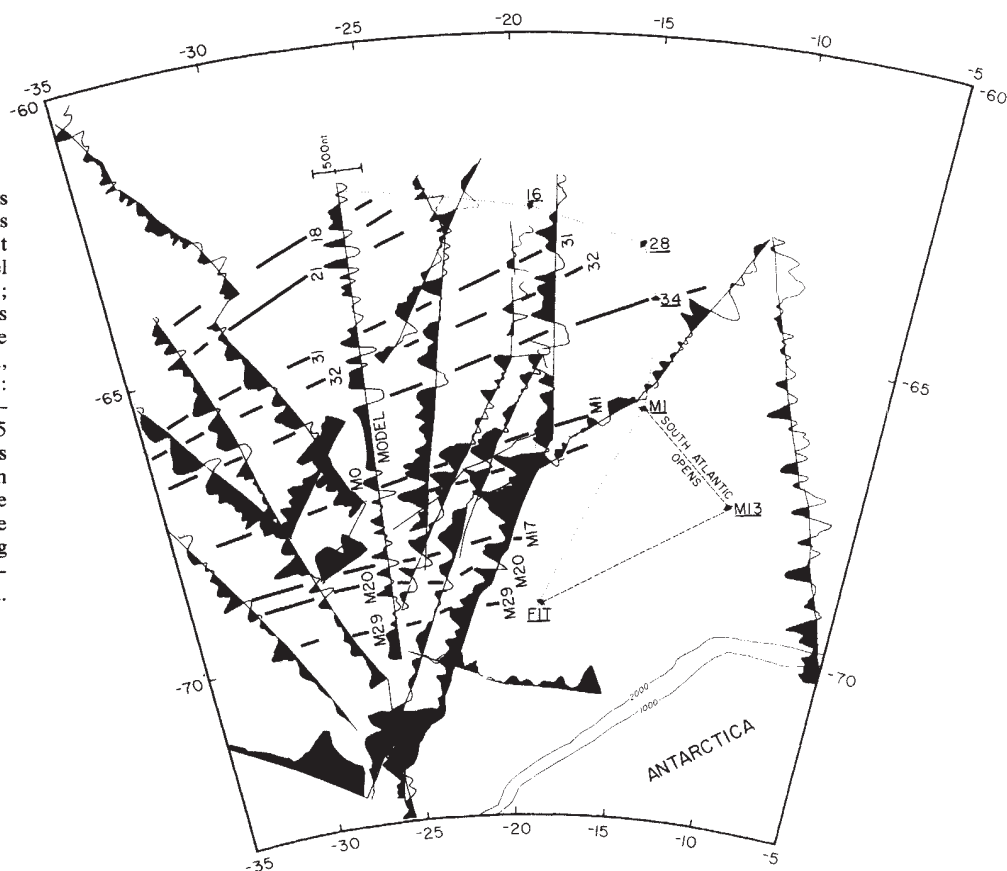
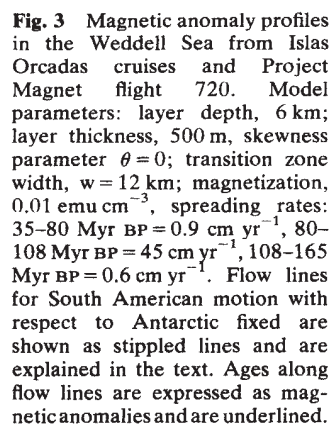


Fig. 2 Line drawings taken from Islas Orcadas seismic reflection profiles located in Fig. 1. The locations of identified magnetic anomalies are indicated below each profile. Station numbers are from ref. 26.



Heat flow measurements taken on board *Islas Orcadas* cruise 1578 reveal a general northerly increase in heat flux within the Weddell Basin²⁶. High heat flow values observed within the Weddell Basin have led to proposals that the Weddell Basin may have formed as a marginal basin during Tertiary times²⁶. The data presented above strongly suggest a much older age for the Weddell Basin. In Figure 4 ages are assigned to the Weddell heat flow stations using the age model presented in Fig. 3, and the Weddell heat flow stations are compared with the global mean values compiled by Parsons and Sclater¹⁴. The high values observed at stations 55 and 40 have been discussed by Zlotnicki *et al.* and may be accounted for by local basement peaks (station 55) and the general variability of individual heat flow values.

The data and model presented above is a first step in tracing the separation of South America and Antarctica since the

breakup of Gondwanaland. Our interpretation of the geophysical data including fracture zone trends, depth to basement, magnetic anomaly lineations, and heat flux indicates that the southern Weddell Basin is pre-late Jurassic to Cretaceous in age. Basement age becomes progressively younger to the north and is Cenozoic in age north of Zone W. We conclude that the Weddell Basin in the area studied was formed at the southern flank of a spreading centre. The northern flank has probably been subducted beneath the Scotia Sea^{12,27,28} or parts may be trapped within it⁶. The general conformity of the observed spreading pattern with that predicted along several lines of evidence for South America–Antarctic relative motion suggests that the spreading centre formed the South American–Antarctic plate boundary throughout much of the breakup of Gondwanaland. The westward convergence of the Weddell magnetic lineation

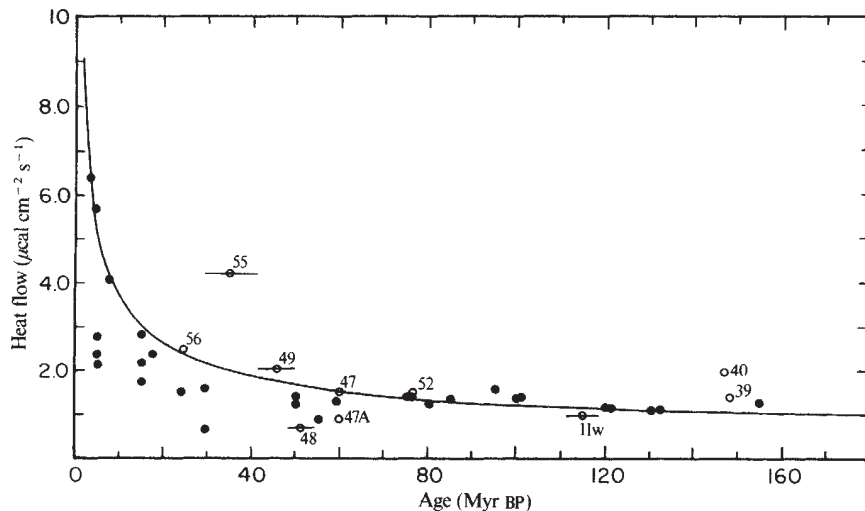


Fig. 4 Comparison of Weddell Basin heat flow values (○) with the Parsons and Sclater heat flow curve (●) using the proposed basement ages.

pattern suggests that the poles of rotation which describe the separation of Antarctic–South America are located very near the Antarctic Peninsula.

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Transcurrent faulting and the anomalous position of pre-Carboniferous Anglesey

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The marked differences of pre-Carboniferous geology between Anglesey and of mainland North Wales are accounted for by Devonian transcurrent faulting probably caused by stresses resulting from the closure of the Iapetus Ocean. Displacement along this fault is thought to have exceeded 100 km, so that the Lake District and not Anglesey would formerly have been in juxtaposition with North Wales.

The Carboniferous Limestone of Anglesey can be closely correlated with that of the North Wales mainland¹, so that in Viséan times the two areas probably had the same geographical relationship as today with continuity of sedimentation between them. There is no reason to suppose that the underlying Carboniferous Basement Beds of the two areas do not have a similar relationship. This is in accord with the concept of a Lower Carboniferous sea encroaching southwards from the Irish Sea Basin onto a landmass of older rocks².

These older rocks present a very different picture. The Precambrian of Anglesey consists of a bedded series of arenaceous and argillaceous sediments with subordinate basic volcanics—the Mona complex^{3,4}—ranging in metamorphic facies from greenschist to amphibolite. The only comparable rocks on the mainland are those of southwestern Llyn⁵ and of the River's Mouth Formation at Caernarvon^{3,6}. The Precambrian of the mainland—the Arvonian—consists essentially of acid and intermediate volcanics (of the Bangor and Padarn ridges⁶ and of Brynteg Borehole⁷ in the Harlech Dome), which have suffered only a low degree of metamorphism. Unless these rocks are represented by the Baron Hill Beds and the Bwlch Gwyn

Felsite^{8,9}—an unlikely correlation—the Arvonian seems to be absent in Anglesey.

Greenly³ provisionally assigned several small outliers in Anglesey, including those of the Careg-onen Beds and the Trefdraeth Conglomerate, to the Cambrian, but later¹⁰ considered them to be older. The only fossils recorded in Anglesey that may be of Cambrian age are stromatolites^{11,12} and microfossils¹³ from the Gwna Group, supposed by Greenly³ to be Precambrian rocks. The rocks containing the stromatolites, however, are associated with mélangé and therefore may be derived blocks in much younger deposits. Indeed, there is stratigraphical evidence (unpublished) showing that at least part of the Gwna Mélangé is of Ordovician age. Whether Cambrian rocks are absent in Anglesey or whether they are represented in the Mona Complex (as the above evidence and the stratigraphical interpretation of Barber and Max¹⁴ suggest), there is a marked contrast with the mainland where, only a few kilometres away across the Menai Strait, are some 2,000–3,000 m of slates and grits. Moreover, these latter rocks apparently overlie the Arvonian conformably or with no great unconformity.

The Ordovician rocks of Anglesey, ranging up to basal Caradoc¹⁵, differ from those of the mainland in several major respects. The generally black mudstones of the former contrast with the predominantly grey silty mudstones of the latter, and there is a greater development of arenaceous rocks in Anglesey. Volcanic rocks, which form such a significant part of the mainland Ordovician sequence, are unknown in Anglesey with the probable exceptions of the Fydlyn Felsite¹⁴ and the relatively thin lavas and tuffs underlying the Middle Llandovery sediments at Parys Mountain¹⁶. Mélanges, a feature of the Ordovician of Anglesey, where they range in age from Arenig to Caradoc, have not been recorded elsewhere in North Wales. In contrast to the mainland, there is evidence of a major pre-Caradoc unconformity in Anglesey¹⁵. This may extend throughout the island, but is best demonstrated in the north, where Caradoc sediments apparently rest on Llanvirn, Arenig and Monian rocks. Upper Caradoc and Ashgill rocks are absent in Anglesey, with the possible exception of the thin volcanics separating Llandovery (*M. argenteus* Zone) and Llanvirn (*D. bifidus* Zone) shales at Parys Mountain. The Ordovician shelly faunas of Anglesey, like those of south-east Ireland and Ramsey Island, Pembrokeshire, have Celtic (Baltic) affinities, whereas those of mainland Wales belong to the Anglo-French (Anglo-Welsh) province^{17–20}.

The only known Silurian rocks in Anglesey are ~50 m of shales at Parys Mountain, attributable to three graptolite zones of the Llandovery. Unlike the equivalent rocks on the mainland, these contain bands of lithic tuff¹⁶. Assuming that the volcanics underlying the Llandovery rocks at Parys Mountain are of Ordovician age, the lower part of the Llandovery sequence of the mainland, which is conformable on the Ordovician, is absent in Anglesey.

The Old Red Sandstone is absent in North Wales except in Anglesey, where it is represented by some 500 m of conglomerates, sandstones and siltstones, probably belonging to the southern British development²¹.

The structure of the Lower Palaeozoic rocks of Anglesey is clearly related in both form and trend to that of the basement. There is evidence that Ordovician sedimentation was controlled by renewed movement on structures within the Mona Complex¹⁷. Although the basement has not been proved on the mainland, it seems most unlikely that the structure of the Lower Palaeozoic rocks reflects that existing in the basement at depth.

This account of fundamental geological differences between Anglesey and the mainland points out the anomalous position of the island with respect to the rest of North Wales in pre-Carboniferous times, suggesting that the two areas did not come into juxtaposition before the late Devonian. So far as the Precambrian is concerned, separation into an oceanic and a continental plate can be effected by a subduction zone—along the Penmynydd Zone of Metamorphism in southeastern Anglesey. Activity on such a zone can perhaps be extended to Tremadoc times—to the end of the Avalonian orogenic

event^{7,22}. During the Ordovician and Silurian periods, however, the operative subduction zone must have lain much farther to the north-west, beyond Anglesey and the Lake District. Closure of the Iapetus Ocean is invoked to account for faunal contrasts between the Ordovician Scoto-Appalachian and Celtic provinces, but the differences between Anglesey and mainland North Wales, belonging respectively to the Celtic and Anglo-French provinces, have not been satisfactorily explained²⁰.

Plate tectonics alone, therefore, do not seem to provide a solution to Anglesey's anomalous position, and it is suggested here that the answer might be found in transcurrent faulting. A northeasterly trending dextral fault with a lateral movement of at least 100 km is envisaged in the vicinity of the Menai Strait (Fig. 1). Such a fault would have a greater displacement than the parallel sinistral Great Glen Fault, but be of lesser magnitude than the subparallel dextral fault postulated²³ along the Iapetus Suture, across Ireland and through the Solway. It has been suggested²⁴, however, that the Great Glen Fault may have undergone up to 100 km of post ~400 Myr dextral movement, and there is doubt²⁵ about the magnitude of the movement along the Iapetus Suture.

The credibility of this speculative transcurrent fault between Anglesey and the rest of North Wales depends on finding areas of compatible geology that could have been adjacent to each before the fracture occurred. In the case of the North Wales mainland the appropriate area is the Lake District.

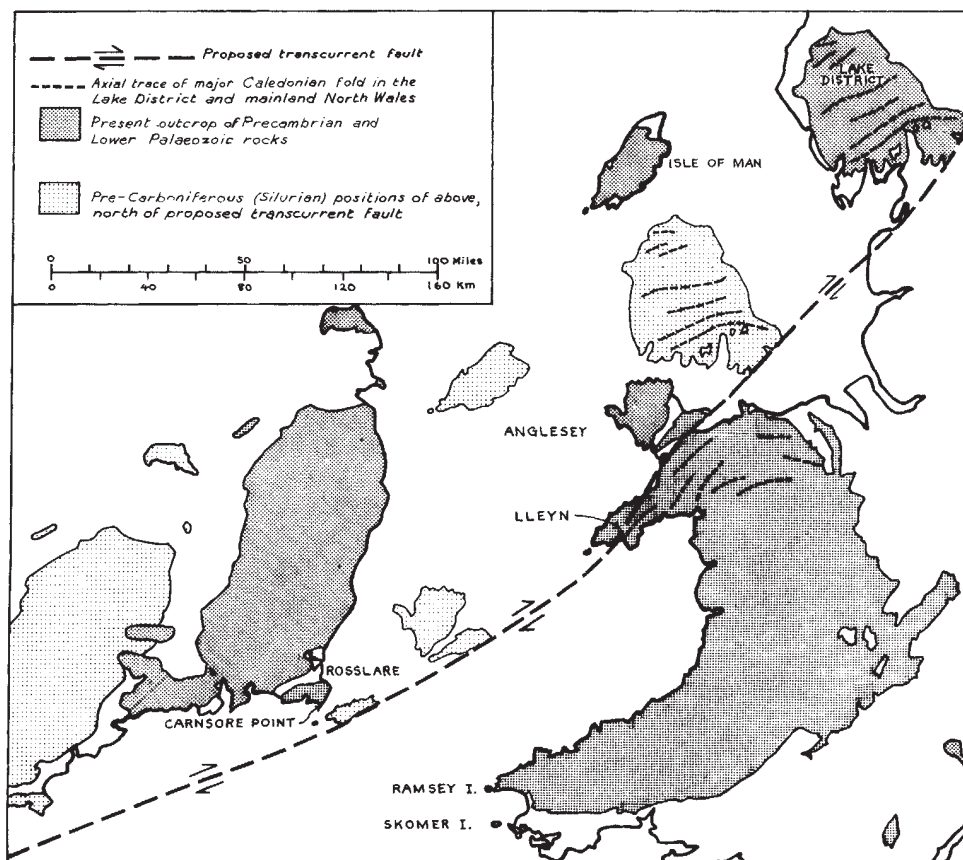
The oldest known rocks in the Lake District are pelitic and arenaceous sediments of the Skiddaw Group, ranging in age from Arenig to Llanvirn. These are comparable to sediments of similar age in North Wales and, like them, contain volcanics—the mid-Arenig tuffs and lavas of the southwestern Lake District²⁶, and the Llanvirn Eycott Volcanics of the northern Lake District²⁷. The Eycott Volcanics are interpreted as the tholeiitic belt of an island-arc environment, and the overlying Llandeilo to Caradoc Borrowdale Volcanics as the calc-alkaline belt²⁸. The Ordovician volcanics of North Wales have been interpreted as part of the calc-alkaline belt in this setting²⁸. Southwards and later this belt gives way to alkaline volcanics. The proposed

transcurrent fault, bringing the two calc-alkaline areas closer together and reducing the width of the belt, as well as eliminating Anglesey from the picture, is advantageous to this model.

Apart from the locally deposited Coniston Limestone and associated beds at the base, the rest of the Lower Palaeozoic sequence in the Lake District is closely comparable with that of mainland North Wales, and it is not unreasonable to suppose that the two sequences were deposited in juxtaposition. The Ashgill Shale Formation²⁹ of the Lake District is lithologically similar to the Bodeidda-Deganwy Mudstones of the Conway area³⁰, the Rebecca Grit of the former³¹ correlating with the Conway Castle Grit of North Wales. The two Llandovery sequences are virtually identical, the Skelgill Beds of the Lake District equating with the succession below the Pale Slates of the Conway area, and the Browgill Beds with the Pale Slates themselves². In the Wenlock there is a marked lithological similarity between the turbiditic Brathay Flags and the Lower Nantglyn Flags, the Lower Coldwell Beds of the Lake District and the Denbigh Grits Group of Wales being proximal facies derived at different times from the west. A prominent band of calcareous concretions in the *Cyrtograptus centrifugus* Zone near the base of the Denbigh Grits Group² compares with a similar band in the same zone in the Lake District and Howgill Fells²⁹. The Middle Coldwell Beds are lithologically identical to the Mottled Mudstones of the Denbigh area, the upper leaf of the former²⁹ having the same zonal position (*Monograptus ludensis*) as the Upper Mottled Mudstone. In the Ludlow the Upper Coldwell Beds equate with the Upper Nantglyn Flags, and the overlying Coniston Grits and Bannisdale Slates are the recognizable equivalents of the lower and upper parts of the Elwy Group respectively. The Coniston Grits are derived dominantly from the north-west²⁹, whilst the slump sheets with sandstones at the same horizon in Wales come from the west². Higher Silurian horizons exposed in the Lake District are not known in North Wales.

The trends of the Caledonian folding in the Lake District and North Wales, swinging from SW-NE to W-E towards the east, show a better fit when the two areas are in north-south

Fig. 1 Map showing the proposed transcurrent fault and the pre-Carboniferous (Silurian) positions of Anglesey and the Lake District relative to Wales. The line of the fault approximates in the north-east to the Dent Line, passing southwestwards beneath Carboniferous and later cover to run along the Menai Strait (the Dinorwic Fault), where transcurrent movement has previously been deduced¹⁴ from the extensive development of mylonites. The fault crosses the Lleyn (where it may have been dislocated by subsequent transcurrent faulting) from Porth Nefyn to the western side of Hell's Mouth, confining the Precambrian rocks, which have affinities with Anglesey, to its north-western side and separating substantially different Ordovician sequences^{5,36-38}.



juxtaposition than in their present geographical positions (Fig. 1).

If the Lake District and the North Wales mainland are matching parts of an Ordovician island-arc sequence and of a Silurian basin of sedimentation torn apart by the proposed transcurrent faulting, we must look southwards for the area originally adjacent to Anglesey, if it still exists. The obvious candidate is south-west Wales (Pembrokeshire and Carmarthenshire). Here, although the Cambrian is markedly unconformable on the Precambrian and there are Ordovician faunal similarities between Ramsey Island and Anglesey as well as agreement between the heavy mineral assemblages of the Old Red Sandstone of Pembrokeshire and Anglesey²¹, the general affinities of south-west Wales are with the North Wales mainland. Thus the Uriconian-type volcanic sequence composing the Precambrian of Pembrokeshire compares with the Arvonian rather than the Mona Complex, there is a thick Cambrian succession, an Ordovician sequence containing limestones, thick volcanics and no recorded mélanges, and, in west Pembrokeshire, a well developed Silurian succession extending through the Wenlock and Ludlow into the Downtonian.

Anglesey is more comparable with southeastern Ireland. The Precambrian para- and ortho-gneisses with mylonites of the island and of the Lleyn Peninsula¹⁴ are not dissimilar from those of the Rosslare Complex³². The basic intrusions forming the Rosslare dyke swarms³³ may be represented in Anglesey by basic dykes cutting gneisses, for example, at Treferwyd¹⁴. The Coedana Granite of Anglesey and the Sarn Granite of the Lleyn belong to the same orogenic episode as the Carnsore Granite intruded into the Rosslare Complex³⁴. The New Harbour Group of Anglesey is similar to the ?Precambrian Cullenstown Formation¹⁴ and the South Stack Formation to the Lower to Middle Cambrian Bray Group³. As in Anglesey, there is a pre-Caradoc unconformity in southeastern Ireland, though the overlying rocks of the latter, the Duncannon Group, are predominantly volcanic³⁵. There is insufficient detailed knowledge of the Llandoverly rocks of southeastern Ireland to attempt a comparison with Anglesey. Structural trends (east-northeasterly to northeasterly) in this part of Ireland are similar to those in Anglesey. The form of the folds in the Irish Lower Palaeozoic is also reminiscent of the situation in the Monian and Ordovician of Anglesey.

The line of the proposed transcurrent fault is shown in Fig. 1.

South-west of the Lleyn the fault probably runs down St George's Channel to lie off the coast of south-east Ireland (Fig. 1), but possibly swings farther west through Rosslare Harbour and along the mylonite zone of the Rosslare Complex^{14,39,40}, separating the gneisses of the latter from the rest of Ireland. An offshore position is favoured, however, because of geophysical evidence of a major fault in this region^{41,42} and the likelihood that the northern boundary of the subsequent Celtic Sea Basin⁴³ corresponds to the line of such a major pre-existing fault^{39,40,44}.

A dextral movement of at least 100 km is envisaged along the proposed fault (Fig. 1). This movement not only removes Anglesey from its present incongruous proximity to North Wales, but creates a more rational relationship between the latter area and the Lake District. A more southwesterly location for Anglesey would also bring the Silurian tuffs of the island closer to the zone of known Silurian volcanism extending from the Dingle Peninsula, through Skomer, to the Mendips⁴⁵. Similarly a more southerly position for the Lake District diminishes the inclination of a southeasterly dipping Lower Palaeozoic Benioff Zone originating along a trench approximately through the Solway. K₂O/SiO₂ plots for Borrowdale volcanic rocks (unpublished data) suggest, following studies of potash-depth relations⁴⁶, that the depth of the Benioff Zone below the central Lake District was of the order of 235 km. This gives a dip from the Solway (allowing for 50% crustal shortening⁴⁷) of ~65°, an excessive figure in comparison with that quoted⁴⁶ for modern continental margin arcs (~40°). The re-location of the Lake District as in Fig. 1 gives a Benioff Zone dip

of ~45°, which angle would be further reduced by a more southwesterly Ordovician location.

The cause of the dextral transcurrent movement can be looked for in NW-SE compressional stresses following the final closure of the Iapetus Ocean in Devonian times, the same explanation invoked²³ for similar lateral movement on the line of the Iapetus Suture itself. Major transcurrent movements affecting the Caledonides of the British Isles and North America in the mid-Devonian and extending over ~20 Myr are indicated by palaeomagnetic studies⁴⁸.

The Devonian movement may have been merely the culmination of dextral wrenching initiated in the Cambrian or Precambrian. Whilst Fig. 1 shows the best fit of the Lake District and North Wales for the Silurian rocks, there is evidence that a more southwesterly position for the Lake District would be better for a match of the Ordovician rocks of the two areas. The fault might, therefore, date back to the Cambrian or Precambrian and its line might have been dictated by the position of the Penmynydd subduction zone during its active lifetime. This situation would be similar to, for example, the present position in New Zealand, where, at an active plate junction, there is an intimate relationship between the subduction zone forming the Kermadec-Tonga Trench and the subparallel strike-slip Alpine Fault⁴⁹.

It is feasible that major northerly and northwesterly faults in North Wales and northwestern England originally developed as conjugate fractures to the proposed transcurrent faulting. The age (around 355 Myr as shown by K-Ar dating) and direction (ENE-WSW) of the main suites of metalliferous veins in Anglesey (Parys Mountain¹⁶), the Lake District (Vale of Newlands⁵⁰), Central Wales (Plynlymon mining field⁵¹) and Salop (Shelve area⁵¹) suggest that these veins may occupy tensional structures associated with dextral transcurrent faulting of the proposed trend, a situation in keeping with the theory⁵² of hydraulic fracturing.

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Evidence of syndedimentary tectonic movements in the Triassic halite of Cheshire

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During the Triassic, north-west Europe was subjected to tensional stresses which resulted in the formation of a complex system of rapidly subsiding grabens and wrench-faulted basins. This pattern of regional crustal extension, which is part of the larger scale Mesozoic break-up of the Pangean megacontinent, is related to the Triassic opening of the Tethys Ocean in southern Europe and rifting in the Arctic North Atlantic, and is a prelude to the Jurassic opening of the southern North Atlantic¹. Great thicknesses of generally continental sediments, often with salt deposits, accumulated in the Triassic basins, which were mainly avolcanic. In western Britain, a complex series of fault-bounded basins extended from the Western Approaches and Channel area northwards to the Irish Sea, and includes the Worcester graben and the Cheshire Basin. The present study of the halite formations in the Cheshire Basin shows evidence for penecontemporaneous fault movements; these were a feature of Triassic times but it is rare that such movements can be detected so precisely as is the case here.

The Cheshire Basin is filled with Triassic sediments dating from the Anisian (lower Middle Triassic) and reaching several kilometres in thickness^{2,3}. There are two thick halite developments in the sequence: a lower Northwich Halite Formation (290 m) and an upper Wilkesley Halite Formation (400 m)⁴. Each of these consists of a series of halite units up to 40 m thick separated from each other by horizons of dolomitic silty clay ('marl'). Between the halite formations, and above and below, there are thick formations of marl of the Mercia Mudstone Group, previously referred to as the Keuper Marl. In the ICI mine at Winsford, Cheshire, where these studies were undertaken, halite is being extracted from two beds in the lower part of the Northwich Halite Formation.

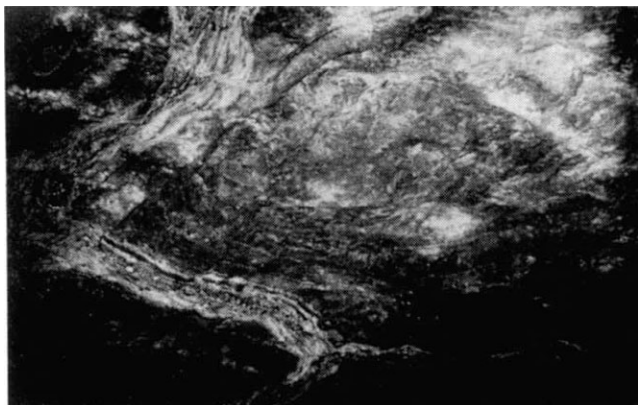


Fig. 1 Photograph of the roof of the mine at Winsford, Cheshire, showing large-scale polygonal pattern (polygon is 14 m wide).

Although principally flat-lying, the halite units in the Cheshire Basin are locally folded into monoclinical structures, which may impart a 45° dip on the beds. The trends of the monoclines are controlled by faults in the underlying Carboniferous basement. Several of the monoclinical structures, trending NE–SW, occur in the area now being mined at Winsford.

The Cheshire halite shows well defined bedding on a scale of 10–50 mm, resulting from an alternation of pure and less pure halite. The salt was deposited from standing bodies of water which were at most only several metres deep⁵. Bromine analysis indicates a marine source for the water (R. M. T., in preparation), although the nearest seawater at the time would have been many hundreds of kilometres distant in southern or northernmost Europe.

On bedding surfaces of the halite, best seen on the roof of the mine, spectacular polygonal patterns are present^{6,7}. These polygons are up to 14 m wide and are defined by 0.5 m-thick bands of secondary salt interlayered with clastic material (Fig. 1). Extending down to 6 m from the polygons, and so seen on mine walls, are tapering V-shaped fissures, with the same layered fills. The layering in the fissures is arranged as a series of

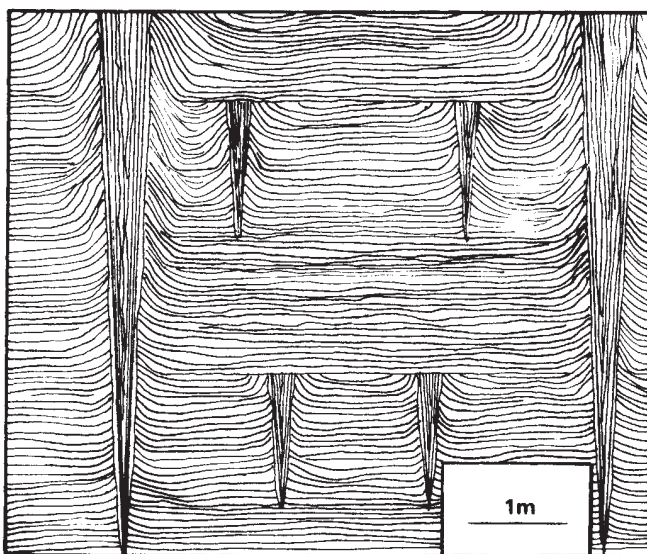


Fig. 2 Sketch from photograph of the wall of the mine at Winsford, Cheshire, showing deep V-shaped fissures and upturned bedding between each fissure.

10 mm wide subvertical bands running parallel to the sides of the fissure. Notably, the fine bands in the fissure fills are paired with a mirror-symmetry about a central unit of secondary salt. Bedding in the halite is upturned adjacent to the fissures (Fig. 2). We propose that each of the clastic layer pairs represents a single opening and filling of the original crack. Capillary brine drawn up the crack, from an underlying ground water table, precipitated the secondary halite. The highly layered nature of the fissure fills thus represents successive openings and fillings of the crack.

The formation of the polygons and fissures is attributed to a process of thermal contraction, acting on the halite after its deposition when it was subaerially exposed⁷. The formation of thermal contraction polygons in halite is very similar to that of periglacial polygons where thermal contraction of ice is involved⁸. Indeed, the thermal properties of salt and ice have much in common, both having high coefficients of linear thermal expansion. When an exposed salt surface is subjected to temperature fluctuations a polygonal pattern, defined by open fissures extending down into the salt bed, is formed during colder periods. The open fissures may then be filled with clastic material transported to the area by wind or water. During subsequent surface temperature increases, the salt surface re-expands and causes compaction of the clastic fissure fill material.

Repeated contraction–crack infilling–expansion of the surface layer of salt causes bending up of the salt beds adjacent to the fissures. The polygons in Cheshire show that there were long intervals of exposure and non-deposition after evaporation to dryness of the water body from which the halite was precipitated.

It can be demonstrated⁷ that the thermal contraction cracks were induced by temperature fluctuations operating over an annual period. Such temperature fluctuations are well documented from modern deserts, including Death Valley, California, where dry salt lakes with polygonal cracks are known⁹.

In some areas of the mine at Winsford, polygons are not developed, but in their place stripes occur. These form two linear belts, trending NE–SW, which can be traced across the whole mine area. The internal layering of the stripes and their associated fissures are identical to those of the polygons and their fissures, so that a process of thermal contraction was also involved. The location and orientation of the linear stripe patterns are significant: they run parallel to the axes of two monoclinical fold structures and are only developed close to the upthrow side of the monoclines, where they replace the polygons (Fig. 3).

Patterns of stripes associated with polygons have been described from several locations^{10,11}. Neal¹¹ demonstrated that stripes would form in sediments subjected to desiccation if they were located on a slope. The sediments would be pre-stressed, because of the effects of gravity, so that on contraction the stress pattern would be mirrored in the pattern of desiccation fissures produced: that is, a striped pattern which trends normal to the dip of the slope. Striped patterns on slopes associated with polygons are also found in the periglacial environment in ice-rich sediments. A process of thermal contraction (but of ice) is also involved, but where stripes form, they are always orientated parallel to the dip of the slope. The reason for this is that ice-rich sediments have the ability to flow downslope. This has the effect of modifying the original polygon form through several phases from stretched polygons to stripes orientated down the slope.

The striped patterns in the Cheshire halite are thus taken to indicate the development of a slight slope on the surface of the halite which must have been the result of contemporaneous movements on the underlying basement faults. That the stripes in the halite are parallel to the monoclinical fold axes, and thus normal to the dip of the slope that existed, shows that the movements on the faults caused the salt above the fault to become stressed, due to the bed being stretched over the fault. When thermal contraction cracking affected the newly exposed salt surface, polygonal patterns formed on the unstressed areas away from the fault–monoclinical axes and striped patterns following the stress pattern formed on the pre-stressed areas above the basement faults.

The thermal contraction polygons and stripes in the Triassic Cheshire halite show that there were contemporaneous movements on Carboniferous basement faults. If the fault movements occurred after thermal contraction cracking had begun, the

resultant crack pattern close to the upthrow side of the fault would be extremely complex, being made up of both polygons and stripes. However, the striped areas in the Winsford mine have a very simple pattern containing only stripes. This then indicates that the fault movements must have occurred either immediately after halite precipitation had ceased, and the salt bed became emergent, or during salt deposition, and it was this movement that caused the salt bed to become subaerially exposed. It is likely that such movements affected sedimentation in the Cheshire Basin in other ways, such as contributing towards the periodic cutting off of the Cheshire Basin from its distant marine water source, and affecting the siliciclastic supply for the basin by controlling rates of uplift of surrounding highlands.

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Functional reconstruction of gait from the Pliocene hominid footprints at Laetoli, northern Tanzania

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Direct measurements of fossilized Pliocene hominid footprints yield only spatial information, such as the length and angle of step^{1–4}. A more complete description of gait requires temporal information so that parameters such as the number of steps taken per minute (cadence) or speed of walking can be calculated. The relationships between step length, cadence and speed of walking have been worked out for modern man and regression equations of high predictability are available⁵. When measurements from the hominid footprints are fitted to these equations a pattern of gait emerges which contradicts previous reconstructions. As we report here, the pattern of gait consistent with the reported footprint measurements from Laetoli is one that might be described as 'strolling', in which the stride is quite short, even when related to the maker's height.

The data reported from Laetoli include the lengths of each foot (*l*) and 'stride', but contrary to the most widely accepted convention the latter actually represent steps or half-strides. Usually stride-length (*L*) is defined as the distance between two consecutive points of contact by the same foot, which means that a stride is made up of two steps. This distinction between step and stride is critical in attempting to reconstruct gait. White⁴ has assessed the stature (*S*) of the makers of the Laetoli footprints; he used data obtained from modern, small people, the Mawabi Pygmies, whose mean foot-length he found to be equivalent to 15.5% of stature. Independent assessment of stature of the East African hominids agrees that they were very small^{6,7}. Thus, from the measurement of stride-length and the estimated stature we can calculate relative stride-length (*L'*).

$$\text{Relative stride-length, } L' = \frac{\text{Stride-length (m)}}{\text{Stature (m)}}$$

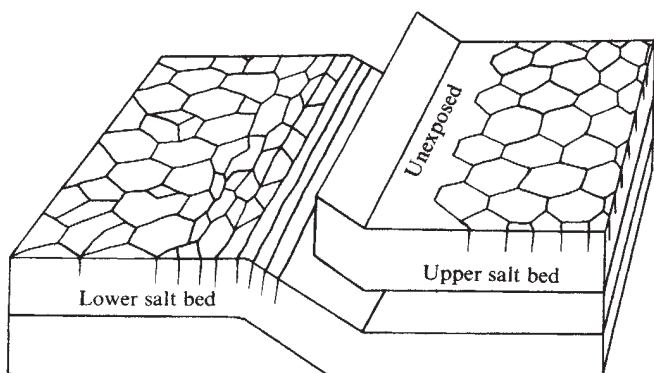


Fig. 3 Schematic block diagram showing the relative positions of polygons and stripes in the ICI mine at Winsford.

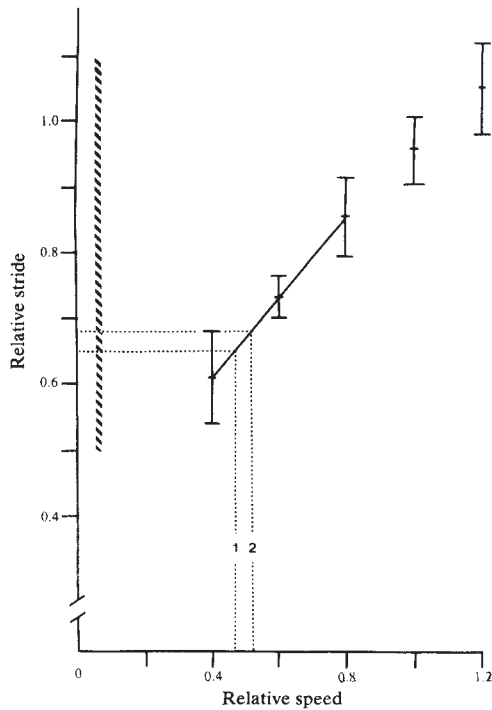


Fig. 1 Graph of stride-length (in units based on fractions of stature) against speed (in units based on fraction of stature covered overground per second) for a normal adult population. In the small portion of the range of relative speeds relevant to this analysis the relationship is linear. The bar on the ordinate reflects the normal range of relative strides available to human adults. The relative speeds of the early hominids whose footprints have been reported indicate a slow traverse across the Laetoli ash beds. Table 1 shows how the ordinate values for hominids leaving trails 1 and 2 are obtained.

Measurements of gait, such as time and length of stride, vary with walking speed. When walking together, at a speed calculated in standard units such as m s^{-1} , a tall and a short subject have very different step-lengths and cadences, the shorter individual having to increase one and/or the other, in order to 'keep up'. These differences in gait due to stature may be factored-out by measuring speeds in units of body length covered overground per second^{5,8,9}; thus

$$\text{Relative speed, } V' = \frac{\text{Velocity (m s}^{-1}\text{)}}{\text{Stature (m)}}$$

Table 1 Gait parameters measured and derived from the Laetoli footprints

Measure	Units	Symbol	Hominid trails		Source or derivation
			1	2	
Foot-length	m	<i>l</i>	0.185	0.215	Direct measurements
Stride-length	m	<i>L</i>	0.774	0.944	Ref. 2
Foot-length-per stride	—	<i>l'</i>	4.18	4.39	
Stature	m	<i>S</i>	1.19	1.39	$S = 100l/15.5$ Ref. 4
Relative stride	Stat	<i>L'</i>	0.65	0.68	$L' = L/S$
Relative speed	Stat. s^{-1}	<i>V'</i>	0.47	0.52	$L' = [60 V'(1 - \beta_2)]/\alpha_2$ Ref. 5
Velocity	m s^{-1}	<i>V</i>	0.56	0.72	$V = V'S$
Stride time	s	<i>T</i>	1.38	1.31	$T = L/V$
Cadence	Steps min^{-1}	<i>C</i>	87	92	$C = 120/T$

The curvilinear equation for predicting *V'* from *L'* is

$$L' = \frac{60 V' (1 - \beta_2)}{\alpha_2} \quad (1)$$

where constants $\alpha_2 = \pm 63$ (males), ± 69 (females), and $\beta_2 = 0.58$.

Figure 1 plots relative speed against relative stride using Grieve's equation and our own data collected from a population of 13 normal young adult caucasian Canadians (6 males, 7 females). Using the measured stride-length and estimated stature, it is possible to calculate values for the relative strides of the early hominids and to use these values to estimate relative speed (Fig. 1).

We use here data from footprints of the two hominids reported by Leakey and Hay² from site G in fossil locality 8 at Laetoli. They assigned the 22 smaller prints to one individual, designating these as trail 1, and the 12 larger prints to a second individual, assigned to trail 2. (Evidently there is far more assurance of these trails than of those found at site A.) The data measured from the footprints at site G, together with the gait parameters derived from them, are shown in Table 1.

Given that modern man normally walks at about 0.86 relative speed^{5,9}, it can be seen from Fig. 1 and Table 1 that the Laetoli hominids walked slowly (around 0.50). Recognizing that in modern man almost every measurable aspect of gait is related to speed, we suggest that in field work the walking speed can be

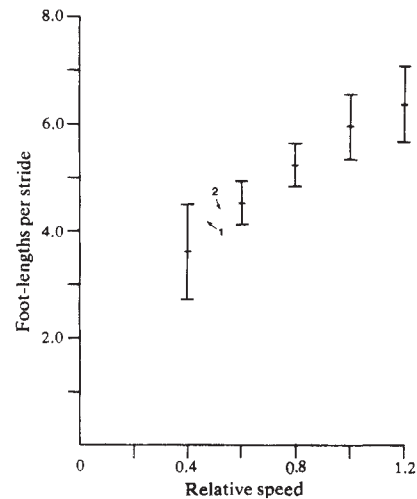


Fig. 2 Graph of stride-lengths in units of foot-length against relative speed, for the same normal young adult human group, showing mean values ± 1 s.d. Based on the relative speed assessment from Fig. 1, the appropriate ordinate values, measured directly off the substrate are plotted, confirming without need of stature estimates of the hominids that their pattern of gait was essentially human.

determined on the basis of the foot-length and the length of stride, which can be measured directly from a set of footprints. Through the most used portion (0.4–1.0) of the range of relative speeds open to modern man there is an approximately linear relationship between the number of foot-lengths in one stride and relative speed (Fig. 2). Thus, even without estimates of stature it is possible to determine whether the gait was slow, medium or fast, and whether footprints made side-by-side are likely to suggest individuals walking along together or traversing the same path but at different speeds and times. Figure 2 confirms our reconstruction of slow speeds, not just slow in human terms, but slow even for these small hominids.

We have not intended to make definitive statements about the characteristic gait of the Laetoli hominids, but rather to show workers in the field how they might reconstruct gait patterns of early bipedal hominids when further trails of footprints are found. Our calculations using data published from Laetoli are based on certain assumptions: that the measurements made on the footprints are accurate, that the prints are indeed those of

early bipedal hominids and that in any sequence of two or three prints only one individual is represented. We used White's estimate of stature⁴, but even using the upper and lower limits from White's tables, and values from groups other than the Mawabi Pygmies our conclusions would not be substantially altered.

If we assume that the relative proportions of the feet of Pliocene hominids are essentially the same as those of modern man⁴, and that their gait was functionally equivalent¹⁰, our determinations of the walking patterns of the hominids (Table 1) are valid, for they are the values that would be expected from modern human subjects of small stature.

Our reconstruction challenges previous claims³ that the stride is "... quite long relative to the creature's small size". On the contrary, it is quite short relative to its maker's stature. Grieve¹¹ has given the adult range of relative strides (Fig. 1) from which it is apparent that a relative stride-length ~ 0.65 is closer to the lower end of the normal range. Normal young adults select 'comfortable' or 'preferred' walking speeds remarkably close to the 0.86 relative speed^{9,12,13}. Grieve^{5,11,14} has pointed out that walking speeds are situation dependent, but we believe that unless some environmental or motivational constraint was acting on these hominids to walk slowly (in the sense of cautiously), the patterns thus far reported from the ashfall tuff of Laetoli are patterns consistent with what humans call 'strolling'. In these conditions there is no need to invoke a narrow step-width or variable step-angle as evidence of a pattern in any way different from that of modern man; normal humans, strolling at around 0.4 relative speed, often exhibit the same features.

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Firefly mate-rivals mimic their predators and vice versa

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Fireflies, beetles of the family Lampyridae, use flashes of bioluminescent light for sexual communication—by means of coded patterns, males and females identify and locate each other in darkness¹. In sexual signalling of *Photinus macdermotti* (eastern United States) the male emits two flashes separated by ~ 2 s and the female then waits ~ 1 s before replying with a single flash (Figs 1a, 2a)². Females of *Photuris versicolor* and *Photuris 'B'* mimic the reply of *P. macdermotti* females thus attracting *P. macdermotti* males, which they eat (Fig. 1b, c). When the predators are decoying *P. macdermotti* males, they sometimes produce 'extra' flashes that mimic the 'competition' flashes used by the males when competing for females (compare Fig. 1d with e)³. I suggest here that the male competition flashes (that the predators mimic) might also be mimics of the predator's own mimicry.

This complex interaction, the apparent product of a long co-evolutionary 'arms' race⁴, is more comprehensible when a model for its evolution is considered (Fig. 3). The predators also

mimic the responses of single-flash species⁵ and it is of special importance to the model that predators became tuned to *P. macdermotti*'s double flash only after they mimicked and preyed on single-flash species. Mimicry mistakes during this evolutionary transition initiated the path to the 'meta-mimicry' proposed here. The success of the tactics of each deceiver, competing male and predaceous *Photuris* female, depends on the influence the other has had, and continues to have on the system. That is, each mimicry provides the basis of support (model) for the other—such reciprocal mimicry is a new phenomenon to science.

The competition flashes (injections) that male *P. macdermotti* inject into the coded dialogues of rivals and contested females are made during the last 0.6 of the rival's flash pattern (Fig. 2b).

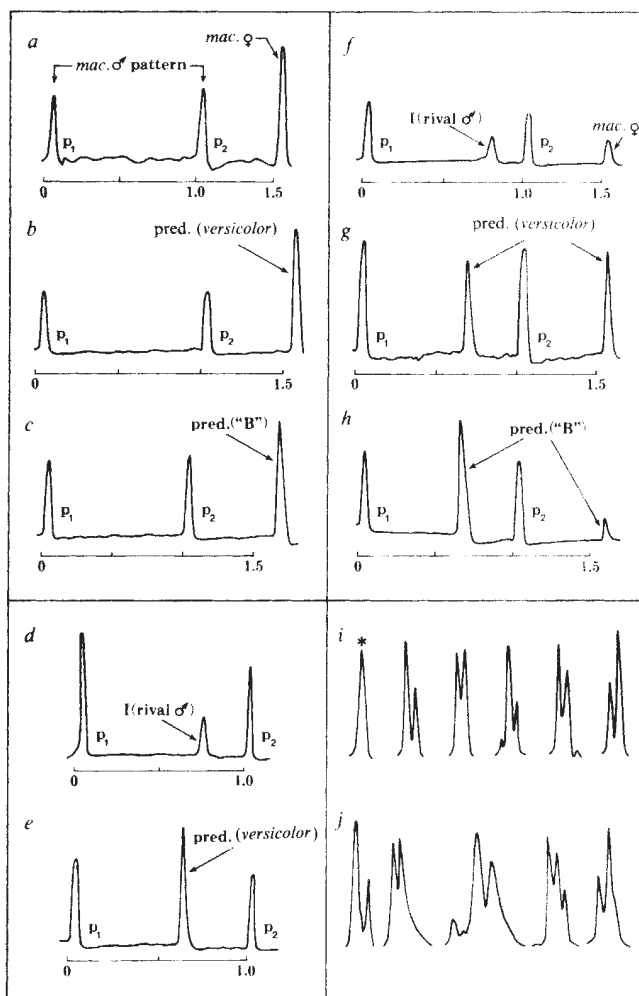


Fig. 1 Chart traces of firefly flashes and interactions, recorded with a photomultiplier system during natural and experimentally manipulated interactions in the field and laboratory. Abscissa represents time, measured as a ratio (in the text) with 1.0 being *P. macdermotti* male p_1 - p_2 interval (this removes temperature effects); ordinate, relative intensity. a, *P. macdermotti* male's characteristic two-pulse (that is, p_1 + p_2) flash pattern, and female answer. b, Simulated male pattern with aggressive mimic *P. versicolor* giving false answer. c, Same as b, with *Photuris* 'B' female giving false answer. d, Male flash pattern with rival injection (I); no female response. e, Simulated male pattern with predator *P. versicolor* giving false injection but no false answer. f, Simulated *P. macdermotti* male pattern, with injection (I) by rival male *P. macdermotti* and answer by female. g, Simulated male pattern with aggressive mimic *P. versicolor* emitting both false injection and false answer. h, Same as g, except aggressive mimic *Photuris* 'B' female. i, Various injection forms of competing *P. macdermotti* males. Note similarity to false answers (j) that aggressive mimics give to single, short flashes (see text).

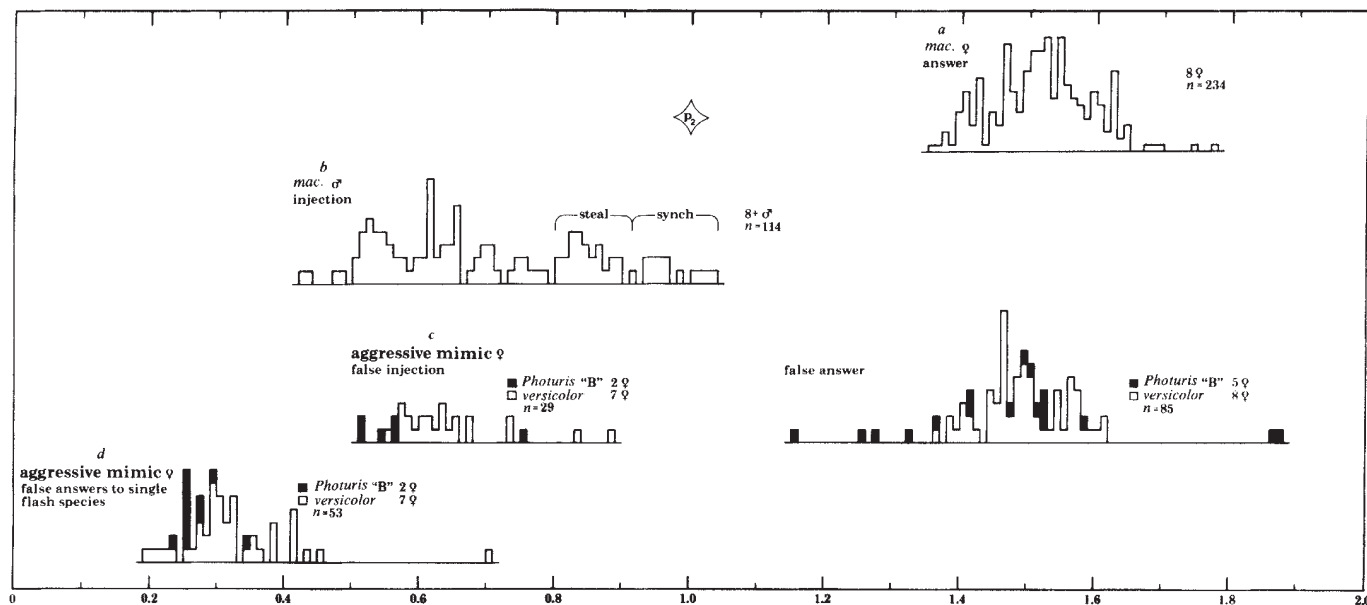


Fig. 2 Time-positioning of various flashed signals and mimics involved in the reciprocal mimicry phenomenon. Abscissa represents time, measured as a fraction of the interval between the two flashes (p_1 and p_2 in Fig. 1) of the *P. macdermotti* male pattern (this corrects for temperature differences among recording sessions as signalling parameters are temperature dependent). The first male flash (p_1) occurs at time 0; the second flash of the male pattern (p_2) occurs at time 1. *a*, Timing of *P. macdermotti* female flash responses, centering ~ 0.5 units after 1 (p_2). *b*, Timing of *P. macdermotti* male competition flashes (injections). 'Steal' and 'synch' indicate other male tactics. *c*, Timing of predators' false injections (mimicking *P. macdermotti* male injections, see *b*) and false answers (mimicking *macdermotti* female answers, see *a*). Note the time correspondence between the false injections in *c* and their model in *b* above; and between false answers in *c*, and their model in *a* above. Note particularly that injections and false injections centre ~ 0.6 units after the preceding male flash (p_1 at time 0), but answers and false answers centre ~ 0.5 units after the preceding male flash (p_2 at time 1). *d*, Timing of the flashed responses used by predators when answering isolated single (non-paired) flashes—presumably the response given to some as yet undetermined, single-flash prey firefly. Note that this response centres ~ 0.3 time units after the 'male' stimulus flash that precedes it.

(Decimal fractions are used to indicate time-positioning because the 'clock-time' of these signals varies with temperature. Unity (1.0) is the mean period between the two flashes in the time-coded pattern of flying, searching males².) Injected flashes take various forms. Those discussed here are simple, like those in the normal flash pattern (Fig. 1*i*, asterisk), and occur between 0.5 and 0.8. The timing of these injections is similar to that of the false injections emitted by both *Photuris* predators (Fig. 2*c*). (Injections occurring later than ~ 0.8 seem to be involved in other competitive, photic tactics; Fig. 2*b*, 'steal' and 'synch'.³)

The false injections (male imitation) and the false answers (female imitation) of predaceous females are two different responses, not merely an identical 'pause then flash' response given after different flashes of the two-flash male pattern. That is, the predators do not simply adopt the same response, but use two different pauses (that is, each matching its own model). Note that false injections (Fig. 2*c*) are centred at ~ 0.6 time units after the male flash that precedes them (p_1 is at 0), and false answers centre at 0.5 time units after the male flash that precedes them (p_2 is at 1.0). Note also the difference in timing of the two in the recordings (Fig. 1*g*, *h*).

The stimulus that elicits the two responses in the predators is the two-flash pattern (the species-specific pattern of *P. macdermotti* males). In contrast, if single flashes are presented at long intervals (>5 s), and therefore a *P. macdermotti*-prey sign-stimulus is not seen by the predator, a different response (pause time) is evoked—this is probably used to prey on another species. In both *P. versicolor* and 'B' this consists of a very short pause, and a modulated flash that is longer than those given in response to *P. macdermotti* patterns (Figs 1*j*, 2*d*). This demonstrates that the false injection is not merely a misplaced false-answer that is correctly used when preying on males of some single-flash species, and it further supports the conclusion that the false injection is a specifically tuned response.

The success of mimics on both sides (predator and prey) depends on the influence the other has on the system. The false injections of *Photuris* must enhance their attraction of prey

because *P. macdermotti* males use their injections to gain advantage over rivals (J.E.L., in preparation), and selection has favoured males that are not duped, but instead continue approaching with caution. The injections of competing males

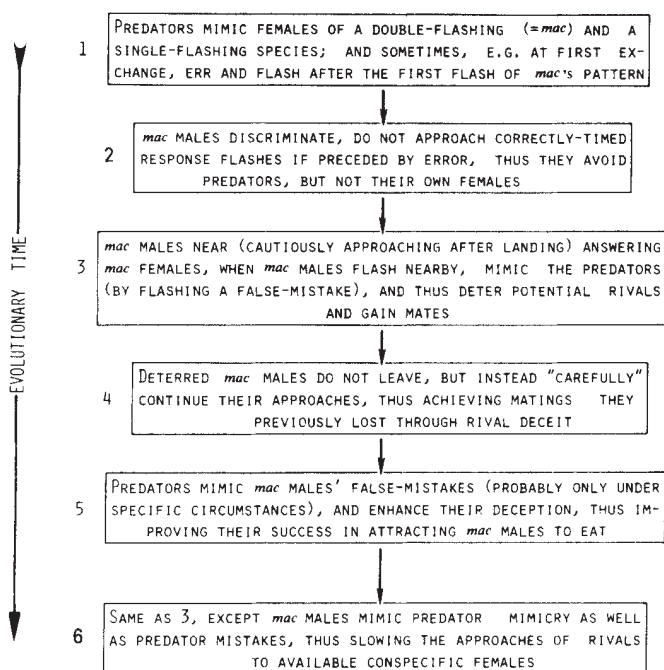


Fig. 3 Model of an evolutionary 'arms' race (conflict coevolution) leading to reciprocal mimicry of *P. macdermotti* males and their predators, *Photuris* females. A number of variants and origins are possible.

continue to influence rival behaviour because *Photuris* females continue to use them to prey on males. An individual of either species that abandons its mimicry is at a disadvantage in its own individual reproductive effort, thus natural selection independently maintains both components of this reciprocal mimicry.

Historical interactions that may have led to this dual mimicry (Fig. 3) will be discussed in more detail elsewhere, but a further possible mimicry is worth noting because it relates to the presumptive evolutionary origin of false injections, mistakes sometimes made by predators. About 75% of the injections made by *P. macdermotti* males during competition are double or triple flashes (Fig. 1i), which resemble flashes made by *Photuris* in response to single-flash stimuli (Fig. 1j). Males emit these modulated flashes only during mate competition. Their strong resemblance to flashes made by *Photuris* further suggests a highly refined mimicry system.

The communicative-deceptive transactions of these fireflies, both predators and mate-rivals, are more complex and finely tuned than expected. Similar refinement and subtlety probably exist in the communicative ecology of most insects, particularly in the contexts of female mate assessment and choice, but escape detection and general recognition because of the technical difficulties that their signals, especially chemical ones, present to discovery and analysis. Fireflies, therefore, offer unique opportunities for investigation of insect communication.

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Removal of bound calcium from nematocyst contents causes discharge

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Nematocysts are the stinging organelles of jellyfish, sea anemones and other cnidarians. Each one consists of a closed capsule filled with fluid. In the resting state, part of the surface of the capsule is inverted, forming a tubular thread, which is everted explosively on excitation. The mechanism of explosion is not yet understood^{1–3}; it may be relevant to exocytosis in general, as the nematocyst is a specialized type of exocytotic secretion originating in the Golgi apparatus⁴. Picken and Skaer¹ observed that the capsular fluid showed a large depression of freezing point, suggesting that the osmotic pressure might be as high as 140 bar. They tentatively ascribed the explosion to a sudden increase in permeability of the capsule wall, allowing a rapid osmotic influx of water. However, there is evidence that the material of the thread itself may be capable of some degree of extension^{5,6} and it has been suggested that osmosis plays no part in discharge³. We present here a new theory: that the capsule wall is permeable to water even in the undischarged state; discharge is initiated by an increase in the osmotic pressure of the capsular fluid which is brought about by removal of bound calcium ions.

The work was carried out on holotrichous isorhizas from the mesenterial filaments of the coralliomorpharian anemone *Rhodactis rhodostoma*. These nematocysts have the advantage that they are large enough (150 μ m long) to be manipulated individually with fine steel needles. All manipulations were carried out in liquid paraffin. Before use, the nematocysts were isolated from the mesenterial filaments by teasing with needles and cleaned of mucus and cell debris by drawing them carefully across a glass surface. Experimental solutions were injected as droplets into the liquid paraffin and the nematocysts transferred from one droplet to the next using a steel needle. Measurements of refractive index were carried out with a Zeiss Jamin-Lebedeff system and with a Baker image-shearing interference system equipped with a fringe-field eyepiece. The freezing point depression of the contents of intact individual capsules was measured by a method to be described in detail elsewhere. It consisted essentially of placing the isolated nematocysts in liquid paraffin inside a sealed chamber which was immersed in a refrigerant of a Ramsay-Brown freezing point apparatus¹. The chamber was viewed microscopically and the formation and melting of ice crystals within individual capsules observed.

This new method of *in situ* measurement revealed slightly higher freezing point depressions than those observed by Picken and Skaer¹, who used nematocysts of *Corynactis* and determined the freezing point of extracted samples of fluid. Our *in situ* measurements on *Rhodactis* gave values for $\Delta^\circ\text{C}$ of -5.4 to -8.2 ($\bar{x} = 6.2$, $n = 15$), whereas those of Picken and Skaer were -4.2 to -5.9 . These values correspond to high notional osmotic pressures, but, as mentioned by Wolf⁷, there is no simple relationship between osmotic pressure and freezing point depression for concentrated macromolecular solutions. Without further knowledge of the chemical composition of the capsular fluid, the osmotic pressure cannot be deduced.

As previously observed⁸, the capsule fluid had a high refractive index. Undischarged capsules gave values as high as 1.378–1.388, corresponding to dry mass concentrations of 25–30% ($n = 4$) in excess over seawater, assuming a refractive increment of 0.0018 (ref. 9). The latter assumption is valid if the capsule solutes are protein, glycoprotein, carbohydrate or amino acids. Capsules which discharged spontaneously underwent a dramatic drop in refractive index over several seconds, which caused the specimens to pass through four orders of interference colour. The final value of refractive index corresponded to a mean dry mass excess over seawater of only 0.56% (s.d. ± 0.16 , $n = 10$) which is probably due to the mass of the capsule wall. It is therefore clear that during discharge there is a massive influx of seawater into the capsule, as observed by others, and that the capsule contents are completely replaced. These observations show that a concentrated and probably highly osmotically active solution in the capsule is drastically diluted during discharge; thus far our findings are consistent with the theory of Picken and Skaer.

Further experiments were performed in an attempt to discover the nature of the process that initiates the influx of water. Attention was directed to calcium because microprobe measurements have shown that the capsule fluid contains calcium at a concentration of about 600 mM per kg wet weight¹⁰. Isolated nematocysts could be made to discharge by placing them in a droplet containing 10 mM potassium citrate¹¹ with 10 mM imidazole buffer, pH 7.0. A similar solution without citrate was ineffective. The excitatory effect was blocked by the addition of 50 mM CaCl_2 to the citrate/imidazole solution (only 1 capsule out of 15 discharged). This blockage was peculiar to calcium, other cations being ineffective: 15 capsules out of 15 discharged in each case when NaCl, KCl or MgCl_2 was used as 50 mM solutions in place of CaCl_2 . The stimulatory effect of citrate is almost certainly due to its chelation of calcium; similar effects were obtained with EGTA. This experiment indicates that calcium ions stabilize the nematocyst in some way (as suggested by Blanquet¹¹) and moreover that their removal may be the cause of discharge.

Table 1 The effects of CaCl_2 and sodium citrate on the tonicity of undischarged capsules

Experiment	No. of shrunken capsules	
	1st solution	2nd solution
CaCl_2 followed by sodium citrate	5	0
CaCl_2 followed by CaCl_2	5	5
Sodium citrate followed by CaCl_2	0	5
Sodium citrate followed by sodium citrate	0	0

In each experiment, groups of five undischarged capsules were placed sequentially for 1 min each in two separate solutions containing 40% w/v polyethylene glycol, 50 mM imidazole buffer, pH 7.0, and either 50 mM CaCl_2 or 50 mM sodium citrate; the state of expansion of the capsules was recorded. A repeat of these experiments with 50 mM MgCl_2 added to the citrate solutions gave similar results. Previously discharged capsules containing only seawater shrank in all the above solutions.

One possibility was that calcium might prevent discharge by rendering the capsule wall impermeable to water. To test this hypothesis, undischarged capsules were placed in a series of stain solutions containing 50 mM imidazole buffer, pH 7.0, and 50 mM CaCl_2 . Capsules were found to be impermeable to a 5% solution of blue dextran (molecular weight (MW) 2×10^6) or to 1% Alcian blue (MW 1,265), even when left for several minutes. However, 1% solutions of phenol red (MW 354), sodium fluoresceinate (MW 376) and bromophenol blue (MW 670) were all able to permeate the capsule, reaching approximately the same concentration as outside within 10–20 s; capsules that had been permeated with stain retained their ability to discharge when placed in a buffered 10 mM citrate solution. These results show that the wall of the undischarged capsule is permeable to charged molecules of relatively low molecular weight. It therefore seems likely that very small molecules such as water can diffuse rapidly in and out of the undischarged nematocyst.

We have considered two other possible mechanisms for the action of calcium. One is that the osmotic pressure of the internal fluid is constant and is continuously exerted; discharge is then made possible by a localized weakening of part of the thread or capsule wall, due perhaps to the loss of calcium as a cross-linking agent. This seems unlikely, as the capsule expands briefly before discharge, as previously observed³, indicating an increase in hydrostatic pressure. An alternative mechanism is that the removal of calcium changes the nature of the capsule fluid such that the osmotic pressure increases.

The latter hypothesis was tested by measuring the tonicity of undischarged capsules with and without calcium in the bathing medium. To prevent discharge, it was necessary to use a solute that could not readily penetrate the capsule wall but was of low enough molecular weight to allow an external solution of relatively high osmotic pressure; polyethylene glycol of MW 1,500 was chosen. Undischarged nematocysts were placed in solutions containing 40% w/v polyethylene glycol, 50 mM imidazole buffer, pH 7.0, and either 50 mM CaCl_2 or 50 mM sodium citrate (Table 1). The capsules shrank when placed in the calcium solution, but re-expanded when placed in the citrate solution. In the reverse experiment, capsules placed first in citrate remained expanded, but shrank when transferred to the calcium solution. These results cannot be ascribed to the increase in the osmotic pressure of the bathing medium due to the addition of the calcium salt, as the addition of 50 mM MgCl_2 to the citrate solutions failed to cause shrinkage. Therefore, these experiments strongly suggest that the tonicity of the capsule fluid in the presence of calcium ions is lower than in their absence.

We propose that the isolated capsule is continuously permeable to water and the internal hydrostatic pressure due to osmosis is sufficient to keep it turgid but insufficient to produce discharge. The normal stimulus for discharge is the removal of calcium from the nematocyst fluid, presumably in ionic form. This results in an increase in the osmotic pressure of the fluid.

The molecular basis of this process is not yet clear; it could involve the dissociation of a macromolecule, creating a larger number of osmotically active particles.

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Immunoradiometric assay of human leukocyte interferon using monoclonal antibody

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Interferon research has been hampered by problems in its assay. The only widespread assays use tissue culture cells and compare some parameter of viral growth (such as viral RNA synthesis or host cell death) in the presence and absence of interferon¹. These complex biological assays, though sensitive, are laborious and subject to inherent variability. In particular, components other than interferon present in the assay sample often influence viral growth. There is clearly a need for a simple, direct, interferon assay; I now describe such an assay—an immunoradiometric assay utilizing monoclonal antibody.

The isolation and properties of a monoclonal antibody (NK2) specific for human leukocyte interferon (Hu-IFN α) were recently reported². This antibody was used to purify Hu-IFN α by immuno-adsorption chromatography. The use of labelled antibody (immunoradiometric assay) rather than labelled antigen (radioimmunoassay) may offer improved assay sensitivity for the reasons given by Miles and Hales³ for insulin assay. Monoclonal antibodies are much more easily purified than are monospecific antibodies from conventional antisera and so will allow the widespread exploitation of the advantages of immunoradiometric assays. For this reason, it was of interest to develop a labelled antibody sandwich assay, in which a sheep anti-interferon antibody⁴ is attached to polystyrene and serves to anchor the interferon present in the sample to the solid phase. The bound interferon is then detected by the addition of ¹²⁵I-labelled NK2 (monoclonal anti-Hu-IFN α) and measurement of the counts bound to the solid phase.

Three forms of polystyrene were used as the solid phase: (1) 3-ml test tubes (LP3, Luckham); (2) 96-well microtitre trays (M24, Gibco); and (3) 6.5-mm beads (Northumbria Biologicals). In the first case the whole tube was counted to measure the bound ¹²⁵I-NK2. When trays were used the bottom of each well was cut off with a hot wire and transferred to a clean tube for counting. In the third case the beads were incubated in 20- or 60-well trays (93-0402, 93-0340, Abbott) and transferred to tubes for counting. The incubation volumes used were 1 ml or 0.1 ml (tubes), 0.1 ml (wells) and 0.2 ml (beads). All three supports were satisfactory and for assaying large numbers of samples the beads were preferred for their convenience.

NK2 antibody was purified from the serum and ascites fluid of mice carrying NK2 tumours by ammonium sulphate precipitation and DEAE-ion-exchange chromatography, labelled by the chloramine-T method and desalted on a Sephadex G-50

(fine) column. The labelled IgG had a specific activity of about $2 \text{ Ci } \mu\text{mol}^{-1}$ or about 1 atom ^{125}I per molecule IgG.

In each assay a standard curve was constructed using either the interferon reference standard MRC 69/19 or a laboratory standard. Such a curve is shown in Fig. 1. The counts bound at $8,000 \text{ U ml}^{-1}$ were at a maximum; no further increase was observed with interferon concentrations up to 10^6 U ml^{-1} . The low level of nonspecific binding ($<1\%$) of input counts is an important characteristic of the sandwich assay.

The assay conditions (Fig. 1 legend) were chosen for the convenient measurement of samples encountered in the purification of interferon, with concentrations of $\sim 10^3$ – 10^7 U ml^{-1} . Solutions containing $>10^3 \text{ U ml}^{-1}$ are diluted by serial dilution and the titration curve obtained matched to the standard curve.

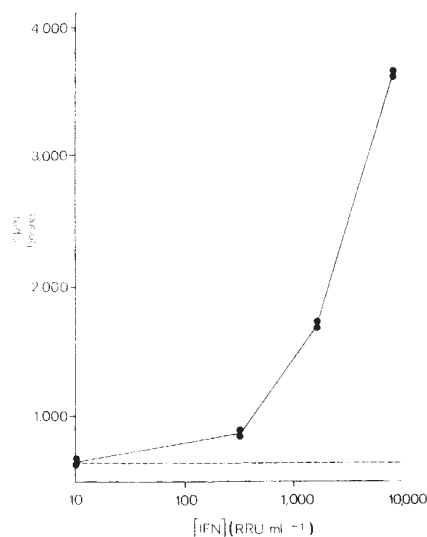


Fig. 1 Standard curve for measurement of interferon concentration by immunoradiometric assay (as obtained by J. Walker). IgG was purified from a sheep anti-Hu-IFN α antiserum⁴ (450,000 neutralizing units per ml) by ammonium sulphate precipitation and DEAE-cellulose ion-exchange chromatography and coated on polystyrene beads by incubation of the beads at 4°C for 16 h in the sheep IgG ($20 \mu\text{g ml}^{-1}$ in PBS, 5 mM EDTA, 0.1% NaN_3). Several hundred beads were coated, washed in PBS, 10% horse serum (Sera-Lab), 0.1% NaN_3 (HS medium) and stored in HS medium at 4°C . Assay trays (20- or 60-well, Abbott) were incubated with HS medium at 4°C to block any sites for protein attachment. Interferon samples were diluted in HS medium and duplicate samples ($200 \mu\text{l}$) added to antibody-coated beads (1 bead per well). After 4 h at 4°C the beads were washed with 12 ml HS medium each using a combined dispenser/aspirator (Pentawash, Abbott). After removal of any residual medium, ^{125}I -NK2 (purified IgG, 40,000 c.p.m.) was added and incubated for 16 h at 4°C . The beads were washed as before and transferred to a γ -counter. The dotted line indicates the background c.p.m. bound when the coated beads were incubated in HS medium without interferon. The IFN concentration is expressed in Reference Research Units (RRU) per ml using the MRC standard 69/19 (National Institute for Biological Standards and Control).

The immunoradiometric assay offers considerable advantages over the conventional biological assays. Since the antibody-coated polystyrene can be stored at 4°C , samples can be assayed at short notice and the results are obtained within 24 h of beginning the assay. The reproducibility, between assays, of the immunoradiometric assay is much better than that of biological assays; four measurements of a solution of $2,000 \text{ U ml}^{-1}$ gave a value of $3,063 \pm 345$ c.p.m. in independent assays in which the input c.p.m. was 47,000–53,000 c.p.m. Only small amounts of sheep antibody are used, since the IgG solution used to coat the polystyrene can be used several times without apparently reducing the sensitivity of the assay. The quality of the sheep antibody may not be a critical factor in the success of the assay,

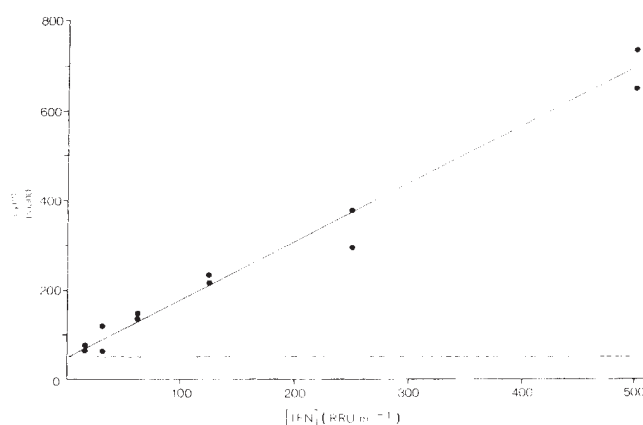


Fig. 2 Standard curve for low levels of interferon. Interferon samples were prepared by dilution of the reference research standard in HS medium and assayed as in Fig. 1 legend except that only 23,000 c.p.m. ^{125}I -NK2 was added to each bead. The dotted line indicates the background c.p.m. bound and was the mean of eight values (s.e.m. = ± 6 c.p.m.).

and other antibodies to Hu-IFN α could probably be substituted. The assay exploits the specificity of the monoclonal antibody NK2, and because this is the product of a hybrid myeloma cell line in culture it can be produced in large amounts without any change in quality. Finally the assay is inexpensive (especially since all the plastic ware except the beads may be re-used), lends itself to automation and several hundred samples can be assayed by one person in a day. Antiviral assays will still be needed to detect any interferons that do not possess the NK2 antigenic site (including IFN β (ref. 5) and possibly some members of the IFN α family) and to confirm biological activity in antigenically positive molecules.

For the measurement of interferon in biological fluids an assay must be capable of detecting much lower levels of interferon than those encountered in production and purification. Figure 2 shows that at low interferon concentrations the counts bound are proportional to the interferon concentration, and concentrations of $\geq 50 \text{ U ml}^{-1}$ can be reliably measured.

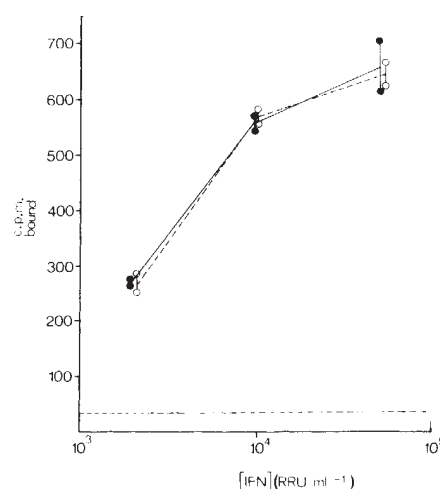


Fig. 3 Interferon assay in the presence of human serum. LP3 tubes were coated with sheep anti-interferon ($100 \mu\text{l}$ per tube, conditions as in Fig. 1 legend). Lymphoblastoid interferon (from stock of 10^5 U ml^{-1}) was diluted in HS medium (●) or undiluted normal human serum (○) and $100 \mu\text{l}$ samples assayed in duplicate by incubating at 4°C for 4 h, washing with $3 \times 2 \text{ ml}$ HS medium and then incubating for a further 16 h at 4°C in the presence of 35,000 c.p.m. ^{125}I -NK2 IgG ($100 \mu\text{l}$). After washing again the tubes were counted for radioactivity.

A limitation in the conventional assay of interferon in serum or other biological fluids has been the need to dilute the sample until the effects of other non-interferon substances that affect viral growth no longer mask the action of the interferon. Dilution of a sample of lymphoblastoid interferon in either phosphate-buffered saline (PBS), 10% horse serum, 0.1% NaN_3 or in undiluted normal human serum resulted in identical titration curves (Fig. 3), indicating that the immunoradiometric assay overcomes this problem.

The lower limit in the measurement of serum interferon by biological assays has been sufficient to detect interferon in the serum of some patients with acute viral infections and in the serum of patients to whom interferon has been administered. This sensitivity is insufficient, however, to measure the interferon (if any) in normal human serum.

It should be possible to improve the sensitivity of the immunoradiometric assay still further by optimizing the conditions at low interferon concentrations. In any case the sensitivity of the assay can be further increased by prior concentration and purification of the interferon on a small NK2 -affinity column.

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Evidence that the serological determinant of H-Y antigen is carbohydrate

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The histocompatibility-Y (H-Y) antigen is a minor histocompatibility antigen which has been detected on cell surfaces from the heterogametic sexes of mammalian, bird, amphibian, teleost and invertebrate species¹⁻³. H-Y is thought to be a male-determining substance in mammals because of its almost perfect correlation with maleness among a variety of mammalian species. To characterize the molecular determinant responsible for H-Y-specific serological activity, H-Y-positive immunoabsorbent cells were first subjected to various treatments which alter protein or carbohydrate structure and then tested for their ability to absorb H-Y antisera. We present here evidence that the serological determinant of H-Y antigen is carbohydrate.

To assess the dependence of H-Y serological activity on polypeptide structure, absorbing cells were treated with the proteases trypsin, α -chymotrypsin and thermolysin. After enzyme treatment and washing, treated splenocytes were used to absorb an aliquot of H-Y antiserum. In this and all subsequent experiments, absorbed sera were tested for cytotoxic activity against male murine epidermal cells. Splenocytes treated with either trypsin, α -chymotrypsin or thermolysin

showed almost the same absorptive capacities as untreated splenocytes (Fig. 1). These results indicate that H-Y-specific serological activity is resistant to these three proteolytic enzyme treatments.

To determine the dependence of H-Y serological activity on carbohydrates, absorbing cells were treated with periodate, which oxidizes 1,2-dihydroxyethyl linkages found in glycosyl residues. Splenocytes were treated with varying concentrations of periodate and washed in a protein+sugar-supplemented medium before absorption. Sensitivity of H-Y-specific absorption to periodate treatment, as well as subsequent experimental treatments, were quantified as corrected per cent inhibition of absorption, as defined in Fig. 2 legend. As a control, the same treatments were performed on absorbing cells bearing antigens of the $H-2^b$ haplotype. Such serological determinants are thought to be contained in amino acid sequences^{4,5}. These data show H-Y-specific absorption to be ~ fivefold more periodate sensitive than $H-2$ (Fig. 2a).

A more direct assessment of the carbohydrate dependence of H-Y serological activity can be made by treating absorbing cells

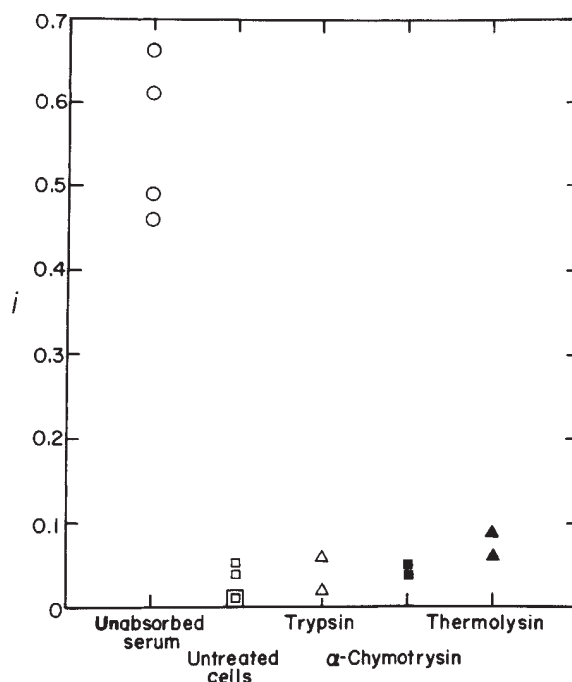


Fig. 1 H-Y-specific absorption: resistance to proteolytic enzymes. Purified trypsin (Gibco), α -chymotrypsin (Worthington), and thermolysin (Calbiochem) were dissolved in Puck's saline F (PSF) to yield individual suspensions containing 0.1% enzyme by weight. Samples containing 4×10^7 splenocytes were incubated in a 2-ml enzyme suspension at pH 7.4, 37 °C for 20 min, washed three times at 4 °C in Medium 199 containing 10% heat-inactivated (56 °C, 30 min) γ -globulin-free fetal calf serum (FCS, Gibco) and used to absorb 25- μ l aliquots of rat H-Y antiserum diluted 1:4 in Medium 199 containing 10% FCS. Rat H-Y antiserum was prepared by the method of Fellous *et al.*⁹ by immunizing female Fisher rats with male (Fisher) splenocytes. Before use in this experiment, rat antisera were heat inactivated (56 °C, 30 min) and absorbed with equal volumes of female murine splenocytes for 1 h at both 0 and 37 °C. An antiserum was regarded as H-Y specific if it had cytotoxic activity against male, but not female, C57BL/6J (B6) epidermal cells, and this activity could be absorbed by male, but not female B6 splenocytes. H-Y-specific immunoabsorption was done on ice (-0°C) for 1 h. Absorbed sera were tested for direct cytotoxicity against B6 epidermal cells according to the method of Scheid *et al.*¹⁰ with the exception that heat-inactivated γ -globulin-free FCS was used as a protein supplement for the isolation medium. Cell killing was quantified by the cytotoxic index, i , defined as: no. dead cells in sample - no. cells killed by complement alone / dose/total no. cells - no. cells killed by complement alone.

with exo-glycosylhydrolases which remove a terminal glycosyl residue from the non-reducing end of a carbohydrate chain. Splenocytes were treated with highly purified β -glucuronidase and β -galactosidase, then washed in protein-enriched media before absorption. Figure 2b shows a graph of corrected per cent inhibition of absorption versus units of enzyme used for treatment. Whereas H-Y-specific absorption is resistant to β -glucuronidase treatment (1,000 units of enzyme destroy 2% of activity from the absorbing cells), it is sensitive to β -galactosidase to the extent that 1,000 units of enzyme remove 54% of the H-Y absorptive capacity of male mouse splenocytes. To confirm that the inhibition by β -galactosidase is not due to uptake of the enzyme by the absorbing cells, the 1,000-unit enzyme treatment was repeated for H-Y in the presence of 0.1 M mannose-6-phosphate, which inhibits the uptake of β -galactosidase by mammalian cells. In the presence of mannose-6-phosphate, the 1,000-unit β -galactosidase treatment still destroyed 45% of H-Y-specific absorptive activity (Table 1). As another control, the 1,000-unit β -galactosidase treatment was used on absorbing cells specific for H-2^b antigens. H-2^b-specific

absorption is completely insensitive to the action of 1,000 units of β -galactosidase (Table 1).

These data suggest that the integrity of an H-Y serological determinant is perturbed by the removal of a terminal galactose from the non-reducing end of an associated carbohydrate moiety. To test further the serological dependence of H-Y on a non-reducing terminal galactose residue, we performed a series of absorbing cell treatments using galactose oxidase, which oxidizes the terminal galactose residue at the non-reducing ends of a carbohydrate chain. Although galactose oxidase has no appreciable effect on H-2^b-specific absorption, it inhibits H-Y-specific absorption by male mouse splenocytes by as much as 38% in this experiment (Fig. 2c).

To verify that the inhibition of H-Y observed here is due to the specific oxidation of a terminal non-reducing galactose rather than to nonspecific oxidation at the cell surface, a similar experiment was carried out using glucose oxidase. Whereas 10 units of galactose oxidase inhibit H-Y-specific absorption by 38%, an equivalent amount of glucose oxidase had only an 8% inhibitory effect (Table 1). This value could possibly be due to

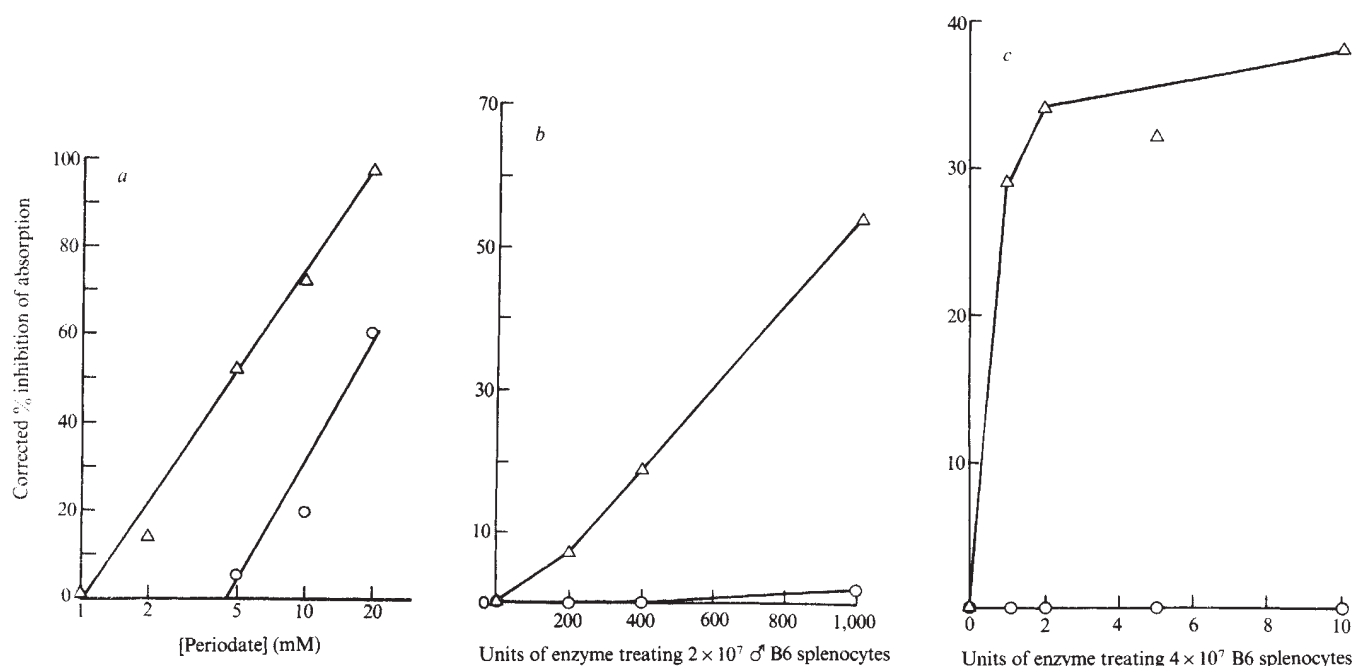


Fig. 2 Sensitivity of H-Y-specific absorption to all treatments quantified as corrected per cent inhibition of absorption, defined as:

$$\frac{i(\text{treated cells}) - i(\text{untreated cells})}{i(\text{unabsorbed serum}) - i(\text{untreated cells})}$$

with i defined as in Fig. 1 legend. Each per cent inhibition value represents the mean of duplicate samples. *a*, Known concentrations of periodic acid (Frederick Smith Chemicals) were dissolved in isotonic phosphate buffer and adjusted to pH 7.4. Splenocytes were treated at a concentration of 10⁷ cells per ml (10⁷ total cells for H-2 and 2 × 10⁷ total cells for H-Y) for 10 min at room temperature (~22 °C). After treatment, the cells were washed twice in Medium 199 containing 10% FCS at 4 °C. Treated splenocytes were used to absorb either mouse H-Y (diluted 1:4) or H-2^b antiserum (diluted 1:100) as described in Fig. 1 legend. Mouse H-Y antiserum was prepared as described by Krcio and Goldberg⁴. H-2^b antiserum was an equivolume mixture of alloantisera to murine H-2.2(D-2) and H-2.33(D-33) specificities obtained from the NIH. Absorbed H-Y antiserum was tested for cytotoxicity as described above. H-2^b antisera were tested for direct cytotoxicity against splenocytes in the following way. Equal amounts (10 μ l) of H-2^b antiserum and B6 splenocytes (8 × 10⁶ per ml) were incubated for 20 min at room temperature; 10 μ l of baby rabbit complement (Pel Freez) diluted 1:4 was then added and the suspension incubated at 37 °C for 35 min. Trypan blue (10 μ l of 0.2% dye) was added for the final 5 min of incubation to stain dead splenocytes. Formalin saline (10 μ l containing 5% formaldehyde) was added as a fixative and live and dead cells were counted. $\circ$, H-2^b; Δ , H-Y. *b*, Highly purified rat β -glucuronidase ($\circ$) and bovine β -galactosidase (Δ) (both 12,000 units per mg protein) were obtained from Dr Virginia Hieber, Department of Pediatrics and Communicable Diseases, University of Michigan Medical School. Splenocytes (2 × 10⁷ total) were treated with known amounts of enzyme in 2.0 ml incubation volumes containing phosphate buffered saline (PBS) at pH 6.8, 37 °C for 1 h. After treatment, cells were washed twice in Medium 199 containing 10% FCS at 4 °C before absorption. Antisera used for absorption and subsequent cytotoxicity testing were murine H-2^b and rat H-Y antisera as described above. *c*, Galactose oxidase (Sigma) was dissolved in PBS containing 1.0 mg per ml of bovine serum albumin (BSA, Sigma). Splenocytes were incubated with known amounts of enzyme in PBS, pH 7.4, at 37 °C for 1 h. Each incubation mixture contained 10⁷ (in the case of H-2^b, $\circ$) or 4 × 10⁷ (in the case of H-Y, Δ) splenocytes plus enzyme at a volume of 0.5 ml. After incubation, treated cells were washed in Medium 199 supplemented with 1.0 M galactose before absorption. Antisera were either murine H-2^b or rat H-Y as described above. Absorption and subsequent cytotoxicity testing were as before.

Table 1 Specificities of enzyme treatment

Enzyme treatment	Total enzyme (U)	% Inhibition of absorption (mean $\pm$ s.e.)	
		H-2 ^b	H-Y
β -Galactosidase alone	1,000	0 $\pm$ 1	54 $\pm$ 8
β -Galactosidase + 0.1 M mannose-6-phosphate	1,000	—	45 $\pm$ 4
Galactose oxidase	10	0 $\pm$ 1	38 $\pm$ 2
Glucose oxidase	10	0 $\pm$ 1	8 $\pm$ 2

β -Galactosidase treatments in the presence of mannose-6-phosphate (Sigma) were as described for Fig. 2b with the exception that 0.1 M mannose-6-phosphate was added to the treatment buffer. Glucose oxidase treatments were as described for Fig. 2c with the exceptions that glucose oxidase (Sigma) was the enzyme used and that treated cells were washed in Medium 199 supplemented with 1.0 M glucose.

the presence of contaminating amounts of galactose oxidase in our enzyme preparation (2% according to manufacturer). In addition, H-2^b was tested for inhibition of absorption by glucose oxidase and no effect was observed (Table 1). These data are consistent with the suggestion that the integrity of an H-Y serological determinant is perturbed by structural alterations directed against a terminal non-reducing galactose on an associated carbohydrate moiety.

The serological reactivity of H-Y antigen, as measured by immunoabsorption, is demonstrated here to be resistant to the action of various proteolytic enzyme treatments. This result implies that the H-Y serological determinant is not a protein, or that it is part of a polypeptide chain resistant to proteolysis. The resistance to proteolysis of H-Y, in combination with its greater sensitivity to periodate than H-2 suggest that the H-Y-specific serological determinant is more likely to be carbohydrate than protein. In most of the experiments reported here, H-2 is used as a control as it is a well characterized glycoprotein antigen whose serological activity is resistant to treatments directed at carbohydrate structures, but sensitive to protein-directed treatments^{5,6}.

We have reported that H-Y-specific absorption is sensitive to both β -galactosidase and galactose oxidase treatments. The sensitivity of H-Y-specific absorption to both these enzymes is in the range 29–54%. Although these values do not reflect complete inhibition, they are consistent with the accepted model of antigen-antibody interaction involving binding of the combining site of an antibody molecule with a carbohydrate determinant of 4–6 glycosyl residues⁷. By perturbing a terminal galactose residue on such a carbohydrate determinant, one would expect detectable, although not complete, inhibition of antibody binding. Thus, our observations of β -galactosidase and galactose oxidase sensitivity indicate a role for a terminal non-reducing galactose residue in the serological integrity of H-Y antigen.

If the serologically active portion of H-Y is carbohydrate, this could account for its evolutionary conservation, suggested by the broad serological cross-reactivity of H-Y-like substances from a variety of species^{1,2,8}. A carbohydrate nature for this determinant could also explain why antisera raised against it are of low titre (1:16–1:32 in the case of our antisera) and affinity. Although the results reported here suggest a carbohydrate nature for the H-Y serological determinant, direct demonstration of whether this immunological determinant is itself a carbohydrate, or is strictly dependent on a carbohydrate chain requires further experimentation.

This work was presented at the meeting of the American Society for Cell Biology in Cincinnati, Ohio on 14–18 November 1980. The abstract of this presentation is published elsewhere⁸.

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Fibrinolysis with acyl-enzymes: a new approach to thrombolytic therapy

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Deep vein thrombosis in man presents a considerable clinical challenge. Despite the availability of prophylactic measures, therapeutic thrombolysis is often necessary, but is difficult and hazardous. Treatments have included the administration of plasmin, other less specific proteolytic enzymes, the indirect plasminogen activator, streptokinase, and the direct activators, urokinase and streptokinase-human plasmin complex. All these treatments have been associated with some haemostatic breakdown, which has discouraged their widespread application. The enzyme components of the coagulation and fibrinolytic pathways can, in general, be classed as serine proteases¹, with a catalytic mechanism which operates via acyl-enzyme intermediates². Chase and Shaw³ showed that *p*-nitrophenyl-*p*'-guanidinobenzoate could specifically acylate the active centre of trypsin-like enzymes, giving rise to a stable *p*-guanidinobenzoyl enzyme and other stable acyl-enzymes have since been described^{4,5}. We report here the fibrinolytic use of acylated derivatives of plasmin (E.C.3.4.21.7) and streptokinase-plasmin(ogen) complexes.

An acyl-plasmin, catalytically inert and therefore unable either to degrade systemic plasma proteins or react irreversibly with its principal plasma inhibitors (α 2-antiplasmin^{6,7} and α 2-macroglobulin⁸), may still bind to a fibrin clot because the lysine/fibrin binding sites on the 'kringle' domains of plasmin are structurally and functionally separate from the catalytic centre⁹ (Fig. 1). Deacylation may then occur to give fibrin-bound plasmin. Wiman and Collen¹⁰ have shown that the involvement of the lysine binding sites of plasmin in binding to fibrin reduces the effectiveness of α 2-antiplasmin and provides part of the molecular basis for selective fibrinolysis *in vivo*¹¹. Thus, if an acyl-plasmin is administered to an animal, the release of plasmin by deacylation of fibrin-bound acyl-plasmin would be expected (within certain kinetic limits) to lead to fibrinolysis. In the systemic circulation, however, α 2-antiplasmin would be expected to act rapidly to neutralize the plasmin released by deacylation, provided that the rate and dose were not so great as to consume the α 2-antiplasmin content of the plasma. The concept of active centre acylation may also be applied to plasminogen activators, provided that they too possess fibrin affinity independent of the catalytic centre. In that case, acylation would allow the activator to reduce interactions with plasma plasminogen which lead, during current therapy in man, to harmful plasmaemia¹². The putative advantages of therapeutic thrombolysis with acyl-plasmins and acyl-activators, therefore, are (1) enhanced efficacy, due to evasion of inhibitor systems; (2) sustained-release pharmacokinetics, leading to simplified administration and easier clinical control; (3) reduction of

systemic toxicity attributable to hyperplasmaemia; and (4) protection of the enzymes against autolytic degradation.

A general method for the specific synthesis of acyl-enzymes was developed using *p*-aminophenyl esters which function as 'inverse' acylating agents¹³. Acyl group radiolabelling demonstrated that active-site acylation with these reagents was quantitative in conditions where nonspecific protein modification was negligible. We prepared acyl derivatives of both human and porcine plasmins, streptokinase-human plasmin activator complex¹⁴ and urokinase, and studied their deacylation rates¹⁵. Using *p*-³H-methoxybenzoyl human plasmin, we found that plasma components on fibrin did not affect the deacylation rate of the acyl-enzyme, and we have confirmed (using immobilized fibrin monomer¹⁶) that active site acylation of plasmin did not affect its affinity for fibrin. Experiments in which *p*-anisoyl human plasmin was mixed with partially purified human α 2-antiplasmin⁷, incubated, re-isolated by affinity absorption on L-lysine-Sepharose¹⁷ and deacylated, showed that an acyl-plasmin did not react irreversibly with the inhibitor. Also, delayed-action fibrinolysis could be demonstrated *in vitro* by incubating human plasma clots with acyl-plasmin, washing and reincubating for a sufficient time to allow deacylation of the absorbed enzyme. Net lysis with acyl-enzymes was significantly greater than lysis by unmodified plasmins.

The kinetics of α 2-antiplasmin action are sufficiently understood¹⁰ to allow the calculation of the steady-state concentrations of free plasmin corresponding to a given rate of deacylation and a given level of acyl-enzyme. Our experimental data in animals have indicated that a steady-state plasma concentration of plasmin amidolytic activity above ~ 10 nM is associated with fibrinogen depletion. It is possible to calculate the relationship between the deacylation rate and dose of an acyl-plasmin that would be consistent with lack of fibrinogen depletion *in vivo*. An acyl-plasmin, at a single dose that would give an initial plasma concentration somewhat less than the known concentration of α 2-antiplasmin in human plasma ($\sim 0.7 \mu\text{M}$)¹⁰, would require a

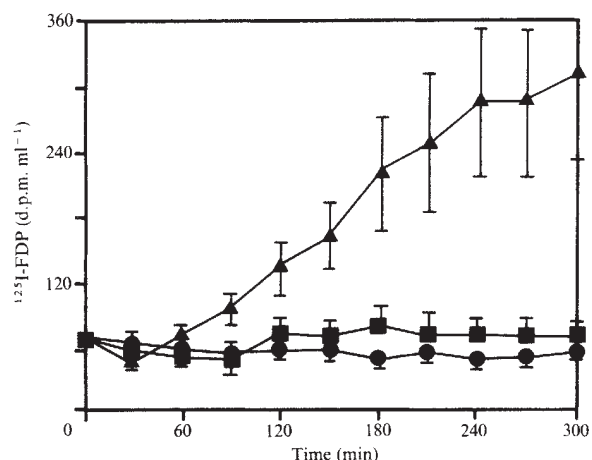


Fig. 2 Release of ^{125}I -fibrin degradation products into the bloodstream of rabbits treated with acylated (▲) and free (■) human plasmins and 0.9% saline solution (●). ^{125}I -fibrin thrombi were prepared in the inferior vena cava of rabbits as detailed elsewhere¹⁵ with the modification that a single strand of 4-ply wool was inserted into the isolated vein segment before thrombus formation to act as a clot anchor. Human plasmin (Kabi batch DtB 23 and Choay batch P299 Pm) was assayed using active site titration³ and amidolytic activity²⁰, and dialysed against 20% v/v glycerol, 0.9% w/v NaCl at 4 °C for 2 h before administration at a dose of 10 mg active enzyme per kg in ~ 20 ml, by constant infusion over 2 h. BRL 26920 was prepared in solution using active site titration³ and amidolytic activity²⁰, and dialysed against 20% v/v glycerol, 0.9% w/v NaCl at 4 °C for 2 h before administration at a dose of 10 mg active enzyme per kg in ~ 20 ml, by constant infusion over 2 h. BRL 26920 was prepared in solution using active site titration³ and amidolytic activity²⁰, and dialysed against 20% v/v glycerol, 0.9% w/v NaCl at 4 °C for 2 h before administration at a dose of 10 mg active enzyme per kg in ~ 20 ml, by constant infusion over 2 h. Complete acylation was checked by amidolytic assay²⁰ and administration was by infusion over 5 min. Control saline, 0.9% w/v, was given over 2 h. ^{125}I -FDP in blood samples was determined by well scintillation counting in a manual bioscint γ counter (Panax) and is given as mean ($\pm$ s.e.m.) concentrations for 12 (BRL 26920), 7 (free plasmin) and 8 (control) animals. Final lysis figures are given in Table 1.

deacylation rate constant of $<1.0 \times 10^{-3} \text{ s}^{-1}$ if the steady-state level of systemic plasmin were to be kept below 10 nM. One can apply kinetic data on the plasmin- α 2-antiplasmin reaction¹⁸ to a calculation of the steady-state concentration of fibrin-bound plasmin necessary to sustain fibrinolysis in the presence of α 2-antiplasmin, using reasonable estimates of the apparent first-order rate constant for breakage of a single peptide bond in the fibrin-plasmin complex, and an estimate of the number of peptide bonds whose cleavage suffices to detach one fibrin monomer from polymeric fibrin¹⁹. Calculation then suggests that a steady-state fibrin-bound plasmin concentration >0.1 nM may be necessary to achieve clot breakdown within a few hours. Acyl-plasmins given at a dose of ~ 0.5 – $1.0 \mu\text{M}$ should achieve this steady-state situation if their deacylation rate constants are greater than $\sim 10^{-5} \text{ s}^{-1}$. Acyl-plasminogen activators require a more complex analysis to take into account the relative rates of activation of plasma and fibrin-bound plasminogen. Control of fibrinolysis *in vivo* by manipulation of acyl-activator deacylation rates could be expected to be correspondingly more difficult, depending in practice on factors such as the plasminogen content of the fibrin clot, and the breakdown and clearance mechanism for both acylated and free activator. After analysis of the steady-state kinetics we concluded that deacylation rate constants of $\sim 10^{-4} \text{ s}^{-1}$ should be consistent with thrombolytic activity for both classes of fibrinolytic agent. Two such agents, *p*-anisoyl human plasmin (BRL 26920), with a deacylation half life of 96 min, and a *p*-anisoyl human plasminogen-streptokinase activator (BRL 26921), with a deacylation half life of 40 min (both measured at 37 °C, pH 7.4), have been selected for further evaluation.

We studied the effects of the two agents compared with saline controls, human plasmin and various streptokinase-plasmin complexes in models of venous thrombosis in the rabbit and the

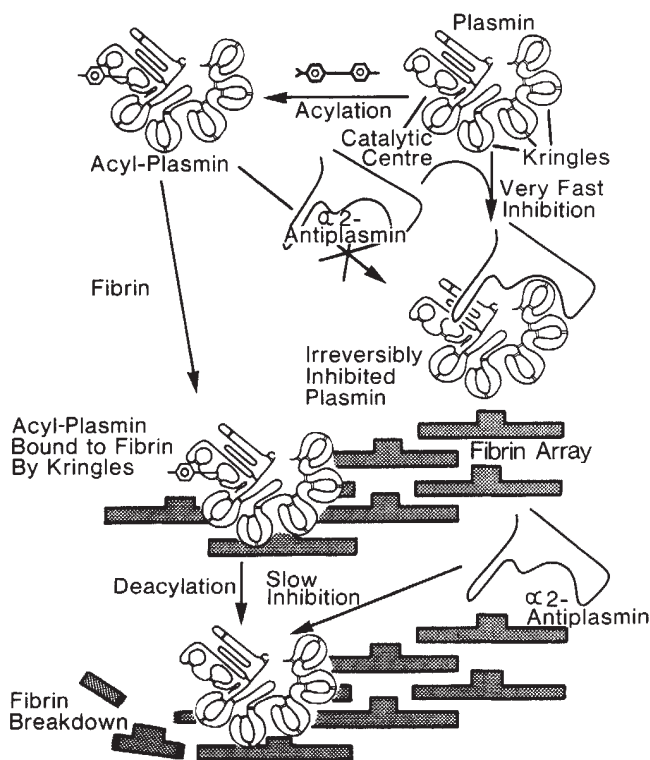


Fig. 1 Schematic outline of the mechanism of fibrinolysis by acyl-plasmins. For description see text. Polypeptide chain structure of plasmin was adapted from ref. 9.

beagle dog (to be published, but partly described elsewhere¹⁵). In both models thromboplastin-induced venous clots radiolabelled with human ¹²⁵I-fibrin were formed around an implanted woollen thread anchored into a major vessel. Lysis was studied by release of ¹²⁵I-fibrin degradation products (¹²⁵I-FDP) into the circulation and by measurement of clot radioactivity at the end of the experiments.

In the rabbit model, acyl-plasmins caused significant thrombolysis when given intravenously as single boluses. Figure 2 shows the effect in rabbits of 10 mg per kg of activator-free free human plasmin (infused over 2 h due to its acute toxicity) and the equivalent bolus dose of BRL 26920, compared with controls given saline. The free plasmin and saline groups showed no significant release of radiolabel into the circulation and gave low, not significantly different, end lysis figures (Table 1). The BRL 26920 group showed sustained release of radioactivity over 5 h and significant net thrombolysis. Free plasmin depleted fibrinogen almost completely, whereas BRL 26920 showed significantly less fibrinogenolysis (Table 1). Plasmin amidolytic activity (chromogenic substrate assay²⁰) in the plasma of animals treated with plasmin was much greater than in animals treated with BRL 26920. The thrombolytic activity of BRL 26920 was also observed in dogs (Table 1).

Because of the known species differences in the mechanism of thrombolysis by streptokinase^{21,22}, BRL 26921 was compared in animals with non-acylated streptokinase-plasmin activator complexes prepared in various conditions (Table 1). Of the unmodified activators, only the minimally degraded form (Type 2) showed significant thrombolytic activity. When degraded activator (Type 1) was *p*-anisoylated at the active centre, the acyl-activator showed increased thrombolytic activity at the equivalent dose. BRL 26921 was tested in groups of rabbits at

three dose levels (Table 1) and showed significant thrombolysis compared with the saline controls. At 2,500 units per kg, all BRL 26921 preparations were consistently more active than all non-acylated activator preparations ($P < 0.01$) and a significant slowing in the rate of ¹²⁵I-FDP release was observed when animals that responded to non-acylated activators were compared with those given acylated activators. None of the tested agents significantly altered fibrinogen levels in the rabbits.

BRL 26921 also showed highly significant thrombolytic activity (measured radiochemically and venographically) against freshly formed thrombi in dogs at two dose levels when compared with saline controls and the least degraded activator form (Table 1). A sustained release of ¹²⁵I-FDP over 4–5 h followed a single dose of BRL 26921. In another group of dogs, the thrombi were aged by isolation from the circulation for 24 h. Isolating ligatures were removed and the dogs treated with BRL 26921 at a single dose of 5,000 units per kg. Radiochemical measurements and venography indicated good thrombolysis (Table 1).

We believe that acyl-enzymes are a novel class of therapeutic agents, in which suitable acyl group modification can provide enzyme precursors whose properties can be manipulated to suit various clinical situations, depending on whether fast or slow thrombolysis is required. Because of their novel pharmacokinetics *in vivo*, they could be expected to show advantages over currently used thrombolytic agents, particularly in producing reduced hyperplasmaemia. We consider that BRL 26921 (which is the more active and accessible agent) may lead to useful improvements in the management of venous thrombosis and some types of arterial thromboembolic disease²³.

We thank Dr J. Cash for advice and help over several years, and also Kabi (Stockholm), Choay (Paris), Novo (Copenhagen)

Table 1 Summary of thrombolytic action of agents in rabbit and dog models of venous thrombosis

Model	Agent	Dose SK units (mg) per kg	No. of animals	% Lysis	% Fibrinogen level	Time to peak ¹²⁵ I-FDP levels (min)
Rabbit	Saline	—	8	3 ± 1	110 ± 1	—
Rabbit	Free human plasmin	(10)	7	7 ± 1	4 ± 3	—
Rabbit	SK-Pm activator Type 1	2,500	5	5 ± 1	95 ± 1	—
Rabbit	SK-Pm activator Type 2	2,500	6	20 ± 4	106 ± 3	49 ± 8
Rabbit	SK-Pm activator Type 3	2,500	5	14 ± 4	—	—
Rabbit		10,000	5	32 ± 5	99 ± 4	36 ± 6
Rabbit	<i>p</i> -Anisoyl activator Type 1	2,500	5	27 ± 7	112 ± 25	162 ± 35
Rabbit	BRL 26921	2,500	9	31 ± 3	95 ± 2	183 ± 32
Rabbit	BRL 26921	5,000	5	38 ± 10	95 ± 2	132 ± 44
Rabbit	BRL 26921	10,000	5	57 ± 11	91 ± 2	144 ± 40
Rabbit	BRL 26921 (lyophilized)	2,500	8	45 ± 6	—	198 ± 23
Rabbit	BRL 26920	(10)	12	21 ± 4	71 ± 8	≥ 300
Dog (short-term)	Saline	—	5	14 ± 5	98 ± 3	—
Dog (short-term)	SK-Pm activator Type 2	1,500	5	10 ± 4	93 ± 5	—
Dog (short-term)	SK-Pm activator Type 2	2,500	5	25 ± 18	107 ± 8	—
Dog (short-term)	BRL 26921	1,500	5	77 ± 14	105 ± 3	312 ± 20
Dog (short-term)	BRL 26921	2,500	5	85 ± 14	109 ± 5	—
Dog (short-term)	BRL 26920	(10)	5	65 ± 9	91 ± 2	330 ± 13
Dog (long-term)	Saline	—	5	38 ± 7	100 ± 7	—
Dog (long-term)	BRL 26921	5,000	5	98 ± 2	100 ± 8	276 ± 78

Activator complexes were prepared from equimolar proportions of streptokinase (SK, Kabi) and lys-plasminogen (Pm, Kabi, K 2086) at a total protein concentration of 1 mg ml⁻¹ in 0.1 M Tris-HCl, 0.9% w/v NaCl, 20% v/v glycerol, pH 7.4. Type 1 activator (degraded) was prepared at 25 °C for 40 min. Type 2 activator (minimally degraded) at 0 °C for 10 min; Type 3 activator was a commercial lyophilized preparation (Behringwerke, Lot 9018) and was dissolved in 0.9% w/v NaCl at a concentration of 1.4 × 10⁵ U ml⁻¹. Acylation of activator complexes was carried out using 1.0 mM *p*-amidinophenyl *p*'-anisate. HCl at 0 °C for 1 h. Other conditions as above. BRL 26921 was prepared in solution by addition of acylating agent to streptokinase, followed by addition of plasminogen and reaction at 0 °C for 1 h. Lyophilized BRL 26921 was prepared on a large scale in solution, diafiltered exhaustively to remove excess acylating agent and lyophilized in the presence of L-lysine, HCl, D-mannitol and human serum albumin. Doses were based on streptokinase contents of activator complexes and administered in 2.0 ml of 0.9% w/v NaCl as a single bolus over 2 min. For plasmin and BRL 26920 dosage and preparation, see Fig. 2 legend. Fibrinogen level was estimated by a modification of the spectrophotometric method of Burmester *et al.*²⁴ at 280 nm. Percentages of the initial level averaged over the experimental period (5 h for rabbits, 6 h for short-term dogs) and over the experimental group. % Lysis, % fibrinogen level and time to peak ¹²⁵I-FDP levels are all means (±s.e.m.). For rabbit model see ref. 15 and Fig. 2. The short-term dog model was similar in outline to the rabbit model, except that thrombi were located in a section of the femoral vein. Homogeneous radiolabelling of blood before clotting around a woollen thread was achieved by aspirating the blood within an isolated vein segment through a collateral vein into a syringe containing radiolabelled fibrinogen. Blood was then returned to the evacuated segment which had been injected with rabbit brain thromboplastin, and restricting ligatures removed after clotting. In the long-term modification of the dog model, restricting ligatures were kept in place to isolate the thrombus from the circulation for 24 h. Treatment was initiated after removal of the ligatures.

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Supposed lamarckian inheritance of immunological tolerance

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The notion that an adaptation acquired during an organism's lifetime can somehow be imprinted on the genome and so become heritable has been faulted by every critical test to which it has hitherto been exposed, but many naturalists have lost their faith in what seems to them to be the all-encompassing explanatory glibness of neo-darwinism. Although this criticism is unfair it is entirely proper that neo-darwinism should be under constant critical scrutiny. Interest was therefore aroused by the claim of Gorczynski and Steele¹ that tolerance of A strain antigens induced in CBA mice by injecting into them (CBA × A/J)F₁ spleen and bone marrow cells could be transmitted down the male line. Such a claim is of particular interest because spermatozoa, unlike oocytes, are produced throughout life and might thus conceivably have participated in the mechanism envisaged by Steele² as that responsible for the supposed transfer of genetic information from soma to germ plasm. It was claimed that as many as 60% of the progeny of tolerant fathers mated with normal CBA females were unresponsive or hyporesponsive in an *in vitro* assay in which their spleen cells were tested for reactivity against A/J strain histocompatibility antigens in the cell-mediated lympholysis assay (CML). We describe here our failure to confirm these findings and our inability to extend them by testing the progeny for their reactivity against skin allografts from the tolerance-conferring strain.

Newborn CBA/Ca (H-2^k) mice (CBA) from six litters were made tolerant of A/J (H-2^a) (A) histocompatibility antigens by the intraperitoneal inoculation of 5 × 10⁷ adult male (CBA × A)F₁ hybrid lymphoid cells, consisting of a 1:10 mixture of bone

marrow and spleen cells. Ten males received the same dose of spleen cells every 2 weeks throughout the breeding programme. The remainder, 13 females and 4 males, received skin grafts from male A strain donors 8 weeks after birth: instead of being sloughed off in 10–11 days, grafts were fully accepted and they remain healthy at the time of writing (11 months later). Of the 10 males selected for breeding, 4 (nos 1–4) received A strain skin grafts 1 week after mating; these animals were likewise fully tolerant. The other six males (nos 5–10) did not receive their skin grafts until the completion of breeding 6 months after birth; these too proved to be fully tolerant.

Each tolerant male was mated with three normal CBA females, and the progeny were marked so that each individual was identifiable throughout the experiment, thus making it possible to compare litters from individual fathers in the two assays used (CML and grafting). A similar procedure was followed in the production of progeny from normal CBA fathers. A total of 201 experimental progeny and 138 normal (or control) progeny were raised; the immunological reactivity of the majority was assessed using the CML assay, and almost all received strain A or (CBA × A)F₁ hybrid skin allografts after completion of the *in vitro* tests. Although Gorczynski and Steele had used only splenic lymphocytes for CML testing, peripheral blood lymphocytes (PBLs) were used by us for a large number of mice because this allowed repeated observations with minimal interference and the transplantation of skin grafts to the same animals. Splenic lymphocytes were used in other experiments; these were obtained by removing a small portion of the spleen, thus ensuring that these animals had splenic tissue at the time of skin grafting. About two-thirds of all hemisplenectomized mice had previously been tested at least once using PBLs. The latter were prepared from heparinized blood drawn from a tail vein, hemisplenectomy was carried out under full Sagatal anaesthesia through a small lateral incision, and skin grafting was done according to the technique of Billingham and Medawar³. Skin grafts were first inspected on day 10 or 11 and thereafter scored daily, necrosis of the whole graft surface providing the end point. Although strain A tail skin was used in the earlier experiments, most of the progeny received (CBA × A)F₁ hybrid grafts because it was felt that this might favour longer survival in hyporeactive animals, a point to which Steele drew our attention.

The *in vitro* sensitization and ⁵¹Cr release CML assay were performed on PBLs as described by Chandler *et al.*⁴. Briefly, PBLs were separated from red cells by hypotonic shock and suspended at 1 × 10⁶ per ml in bicarbonate-buffered RPMI medium supplemented with fetal calf serum, 10 mM HEPES, 5 × 10⁻⁵ M 2-mercaptoethanol, glutamine, penicillin and streptomycin. Aliquots (100 μl) of this suspension were placed in each well of round-bottomed microtitre plates (Sterilin M24

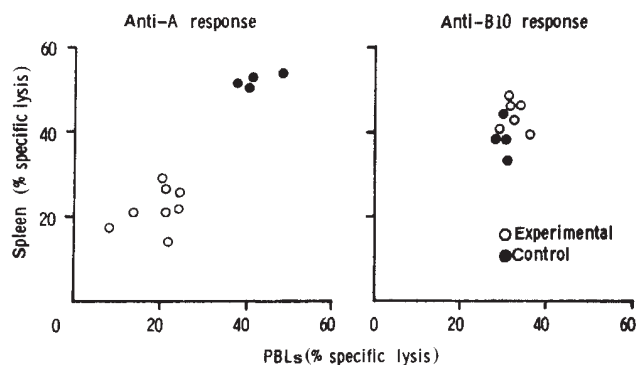


Fig. 1 Cytotoxic responses of experimental and control fathers (spleen versus PBLs). Cytotoxicity is at a K:T ratio of 5:1, derived from regression analysis.

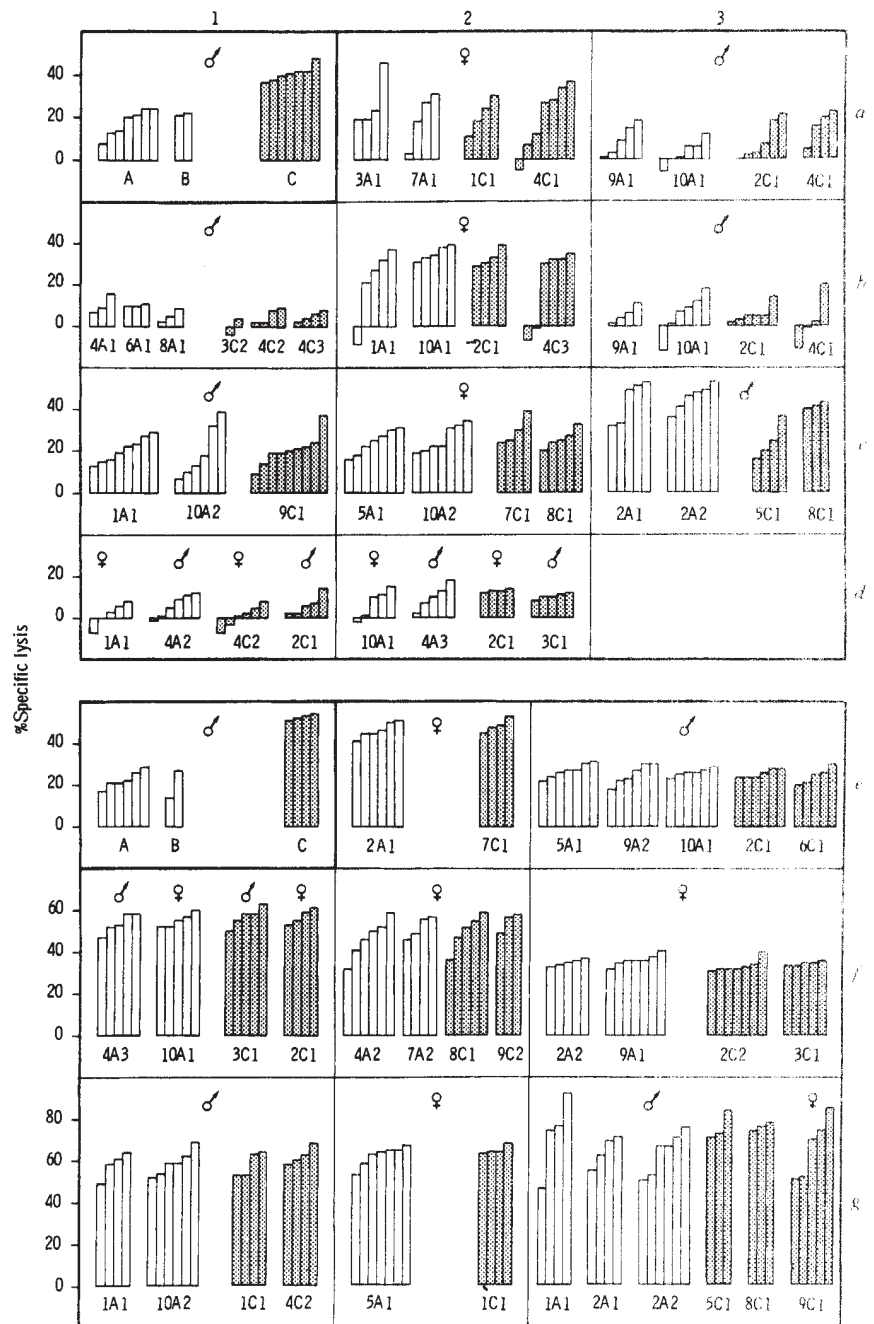


Fig. 2 Anti-A cytotoxic responses of PBLs (panels a-d) and spleen cells (e-g). A, experimental fathers, also shown in Fig. 1. B, males given only one neonatal inoculation of 5×10^7 (CBA $\times$ A) F_1 spleen and bone marrow cells. C, control fathers. The progeny are identified by the number of the father followed by A or C and then the individual litter number. Each panel represents the data from a separate experiment, with sex of the responders indicated. Cytotoxicity is at a K:T ratio of 5:1, derived from regression analysis.

ART) and the same volume and concentration of irradiated (2,000 rad) allogeneic spleen cells were added as antigen. Three to six wells of each PBL sample were set up against the alloantigen being tested, A/J, and whenever PBL recovery had been sufficient (in over 70% of samples), a further three to six wells were set up against 'third party' C57BL/10 alloantigen. After 5 days' incubation at 37°C and in a humidified 5% CO₂ atmosphere, the responder ('killer') cells were collected and the appropriate wells for each sample pooled, counted and dispensed in four twofold dilutions into fresh microtitre plates. 5×10^3 or 1×10^4 ^{51}Cr -labelled 24-h concanavalin A blasts from spleen cultures of A/J or C57BL/10 were then added. The highest killer: target ratio (K:T) was 10:1. The plates were spun briefly and incubated for 3 h before removal of an aliquot of the supernatant for γ -counting. Percentage specific lysis was taken

as $(E - C/M) \times 100$, where E was the c.p.m. for the experimental supernatant, C the c.p.m. for the medium control supernatant and M the c.p.m. of target cells lysed with 5% Triton. Regression analysis of the percentages of specific lysis titrated against K:T ratio was performed and from this curve the percentage of specific lysis at a ratio of 5:1 was obtained. The r^2 value (r = the coefficient of correlation) of each curve was recorded and used in the assessment of the results.

Spleen cells from partial splenectomy were set up in culture in the same RPMI-supplemented medium as the PBLs, but at a concentration of $5 \times 10^6 \text{ ml}^{-1}$. Aliquots (2 ml) of this suspension were incubated in 7-ml Linbro wells (Flow Laboratories 76-053-05) together with 2 ml of irradiated spleen cells ($5 \times 10^6 \text{ ml}^{-1}$) as antigen. The cells were cultured for 5 days and the assay performed as for PBLs, except that the ^{51}Cr -labelled

Table 1 Survival of skin allografts transplanted to experimental and control progeny

Father	Sex	Graft survival time (days)							
		Experimental progeny				Control progeny			
		No.	No CML [†]	PBLs [‡]	PBLs/spleen [§]	No.	No CML [†]	PBLs [‡]	PBLs/spleen [§]
1	♂	10	—	11 13	<11 <11 <11 <11 <11 12½ 13½ 15½	3	—	—	<11 <11 13½
	♀	5	—	—	<10 <10 10 12 12	3	—	—	<11 <11 <11
2	♂	12	<11 16½	—	<11 <11 <11 <11 <11 12½ 12½ 12½ 13½ 14	13	<10 <10	—	<10 10 <11 <11 <11 11½ 12½ 12½ 13 13½ 16½
	♀	14	<11	—	<11 <11 <11 <11 <11 <11 <11 <11 <11 <11 <11 12½ 13½	20	—	<11 <11 <11 <11 <11 <11 <11 <11 <11 12½	<11 <11 <11 <11 <11 <11 <11 <11 11½ 14½
3	♂	6	12½ 15½ 15½	11½ 13 14½	—	9	<10 13	<11 17½	<11 <11 <11 11½ 11½
	♀	6	<11 <11	<11* <11* <11* <11*	—	6	—	<11 <11	<11 <11 <11 <11
4	♂	20	<11	10½ <11* <11* <11* <11* 11 11½ 13½*	<10 <10 <10 <10 <11 <11 <11 <11 14 14½ 14½	16	—	<11 <11 <11 <11 11½ 12½ 13½ 14½	<11 <11 <11 <11 <11 11 11½ 15½
	♀	12	<11	<11* <11* <11* <11* <11*	<11 <11 <11 <11 <11 12½	13	—	<11 <11 <11 <11 <11 <11 12½	<10 <10 <10 <10 <10 11
5	♂	14	11 15½	<11* <11* <11* <11* <11*	<11 <11 <11 13½ 14½ 15½ 16½	3	—	—	<11 <11 <11
	♀	9	—	<11* <11* <11* <11*	<11 <11 <11 12½ 14½	2	<10 <10	—	—
6	♂	7	<11 <11 11½	11½ 11½ 11½ 12½	—	8	10 14½ 14½	—	<11 <11 <11 13½ 15½
	♀	0	—	—	—	1	<10	—	—
7	♂	16	<10 <11 11½ 12½ 12½ 13 13½ 13½ 13½ 14½ 14½	11½ 11½ 12 12½ 14½	—	2	<10 11	—	—
	♀	8	—	<11* <11* <11* <11*	<11 <11 <11 <11	4	—	—	<11 <11 <11 <11
8	♂	6	<11 <11 11½	11½ 11½ 15½	—	2	—	—	<11 <11
	♀	7	<10 <10 <10 11½	<10 10 14½	—	5	—	—	<11 <11 <11 <11 <11
9	♂	5	—	—	<11 <11 <11 13½ 14½	9	<11 <11 <11 12	<11 13 14 14½ 15½	—
	♀	9	<11 <11	—	<11 <11 <11 <11 <11 <11 14½	7	—	—	<11 <11 <11 <11 <11 <11 <11
10	♂	12	—	—	<11 <11 <11 <11 <11 <11 <11 <11 13½ 13½ 14½ 15½	2	13½ 13½	—	—
	♀	15	<10 11½ 11½	<11 <11 <11 <11 <11 13½ 17	<11 <11 <11 <11 <11	2	<10 <10	—	—
Totals	♂	108	25	30	53	67	15	15	37
	♀	85	13	27	45	63	5	19	39
Median survival times of	♂		11½	11½	<11		<11	<11	<11
	♀		<11	<11	<11		<10	<11	<11

Survival times are for (CBA × A)F₁ hybrid grafts except those marked by an asterisk, which were derived from A strain donors.[†] Progeny not tested in CML.[‡] Only tested with PBLs.[§] Tested with PBLs and spleen cells.

target cells were increased to 1×10^5 per well; the highest K:T ratio was again $\sim 10:1$.

Both PBL and spleen samples were scrupulously randomized and coded at the time of their removal from the mice. This code was not broken until after the CML results had been computed.

In most experiments, responders of only one sex were used. Spleen cells from female mice were used for antigen and as targets whenever the responder cell donors were females. Male mice were normally stimulated by, and tested on, male cells but

on a few occasions (and always in experiments where both males and females were used as responders) female antigen and target cells were used. In this way the analysis remained uncomplicated by anti-H-Y reactivity.

The results of testing the tolerant (designated A) and the normal (designated C) fathers in the CML test, using both PBLs and spleen, are shown in Fig. 1 and in Fig. 2, panels 1a and 1e. The two groups are clearly distinguishable from each other by their CML reactivity to A strain, but not to third party B10,

alloantigens. The tolerant CBA fathers were thus specifically hyporesponsive to A strain antigens and this hyporesponsiveness is shown equally by splenic and peripheral blood lymphocytes. Nevertheless, they were considerably more responsive than were the tolerant CBA fathers in Gorczynski and Steele's experiments¹. The reason for this difference cannot be that in our study CML reactivity was tested at a rather late stage (~10 months after birth) because this was also true of the experimental fathers tested by Gorczynski and Steele¹ (8–10 months). It could, however, reflect the fact that the control CBA-anti-A/J response in Gorczynski and Steele's experiments was substantially lower than that in many of our experiments or that, contrary to our practice, their assessment was made with control mice that had not been age-matched. Bearing in mind the advanced age of the experimental fathers, this makes a direct comparison of paternal CML responsiveness in the two studies difficult.

The CML data of the progeny (Fig. 2, panels 2a–3d and 2e–3g) do not show any consistent pattern of hyporeactivity towards A alloantigens. These data include all spleen and PBL experiments which met the technical criteria of (1) the spontaneous medium release of ⁵¹Cr by target cells being less than 40% of maximum release and (2) at least 60% of the responders showing cytotoxic activity which titrated and which, on regression analysis, had an r^2 value of 0.80–1.00. The number of mice tested and assays performed that met these criteria are shown in Fig. 3, which also provides the pooled anti-A results of (1) all experiments performed using PBLs, (2) the data selected from the PBL experiments according to the criteria above and (3) spleen data, all of which met the criteria with the exception of one experiment. It is clear from Fig. 3a–c that there is no essential difference between the response of progeny of tolerant and of control fathers.

However, the pooling of data from many different experiments, each using a different batch of target cells, is not acceptable for statistical analysis, and weight was therefore placed only on the comparisons of animals tested within a single experiment. The data of 20 such experiments are shown as individual panels in Fig. 2. The results of each have been analysed by the Wilcoxon rank order test to determine whether the tolerant fathers or their progeny are significantly different from those of the normal fathers or their progeny. There is a highly significant difference between the response of the tolerant fathers and the control fathers (PBLs, panel 1a, $P < 0.001$; spleen, panel 1e, $P < 0.005$). There is also a difference in the response of experimental offspring in panel 3c (PBLs), with the male progeny of father 2A giving higher responses than the male progeny of control father 5C plus 8C. This difference is significant at the 0.05% level. However, panel 3g shows that the spleen responses of the same experimental mice were significantly lower ($P < 0.01$) than those of the controls 5C plus 8C, although the differences are quantitatively small. In the spleen experiment, moreover, the anti-B10 responses of the 2A progeny were lower than those of the controls 5C and 8C ($P < 0.02$). One further PBL experiment (Fig. 2, panel 3a) shows a significantly lower response ($P < 0.02$) by some of the progeny of father 10A than by some offspring of control father 4C (but not 2C), although this result was not reproducible (see panel 3b). These two positive results cannot therefore be held to carry any conviction—they seem to be non-reproducible and/or nonspecific. The results given in the remaining panels show no significant difference between the responses of experimental and control progeny. In each experiment the progeny of individual experimental fathers were compared with those of individual control fathers tested on the same occasion. All experiments completed but not shown in Fig. 2 because they failed to meet the technical criteria were nevertheless similarly analysed, again without detectable differences. Table 1 shows the numbers of offspring of each sex tested for each of the experimental and control fathers.

Our investigation extended the scope of the analysis described by Gorczynski and Steele¹ in that almost all offspring, including some that could not be included in the CML assay, eventually

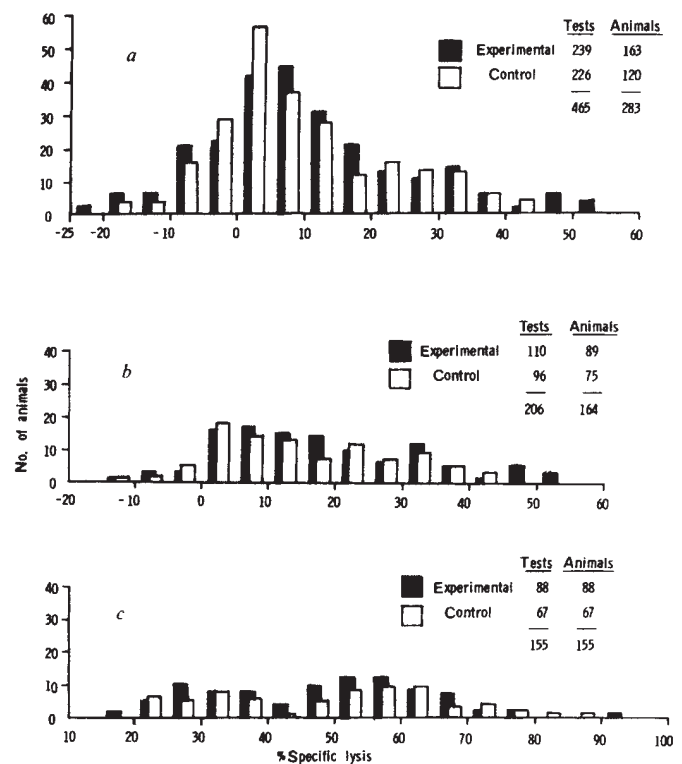


Fig. 3 Anti-A cytotoxic responses of PBLs and spleen-pooled data. a, PBLs, unselected data. b, PBLs, selected data, $r^2 > 0.80$ in $> 60\%$ of mice in each experiment and spontaneous release $< 40\%$. c, Spleen data excluding one experiment. Cytotoxicity is at a K:T ratio of 5:1, derived from regression analysis.

received skin allografts bearing A strain antigens—a total of 193 experimental and 130 control mice. Of these, 80% had been tested for CML reactivity. In addition, the 10 experimental fathers were grafted with A strain skin, 4 of them (nos 1–4) at commencement and the remainder (nos 5–10) after completion of the breeding programme; all these fathers were fully tolerant and carried healthy grafts throughout their subsequent life (up to 11 months). Table 1 gives a detailed breakdown of the individual survival times of grafts transplanted to the experimental and control progeny and it identifies those mice that had been tested in CML and those (the minority) that had not. There is no discernible difference between experimental and control progeny. It is evident that strain A grafts were rejected swiftly and consistently by both male and female recipients, whereas F₁ grafts were rejected more variably, especially by males. The range of survival of grafts on experimental and control mice was closely similar and no graft showed long-term survival.

It is therefore clear that, using the same strain combination, we have been unable to confirm the CML results of Gorczynski and Steele, and the *in vitro* data receive full support from the skin graft assay. Reasons for the discrepancy between the CML data from the two studies can be looked for in the following points.

(1) The initial tolerance-conferring inoculum in our experiments consisted of 5×10^7 cells of a mixture of F₁ hybrid spleen and bone marrow cells, half the number reported by Gorczynski and Steele¹. Furthermore, the ratio of bone marrow cells to spleen cells was 1:10, compared with 1:1 in their study. However, a single dose of 5×10^7 lymphoid cells is certainly adequate for the induction and maintenance of skin graft tolerance and CML hyporesponsiveness (see above and Fig. 2, group B; also ref. 5); indeed, males receiving a single dose at birth had a CML reactivity indistinguishable from that of repeatedly injected males (group B versus group A of Fig. 2, panels 1a and 1e). We would therefore not expect these

differences in protocol to explain the discrepancy if tolerance is the criterion chosen. We have nevertheless begun further experiments in which mice have received 5×10^7 F₁ hybrid spleen and 5×10^7 bone marrow cells at birth, followed by subsequent doses of 5×10^7 spleen and bone marrow cells at a ratio of 1:1. Data from this additional study will not be available for some time.

(2) Although Gorczynski and Steele provide some documentation on the number of experimental fathers and their progeny, there is no information about their controls, which were presumably taken from standard stock without reference to known fathers. This point has been taken care of in our study.

(3) Although the sex of the experimental progeny is clearly shown in Gorczynski and Steele's pedigree diagram, there is no information about the sex of the controls in any one experiment (series), nor is it possible to ascertain what the sex of the stimulator and target cells was. It might therefore be argued that the responsiveness of males and females was different and that their representation in control and experimental groups was uneven, and/or that anti-H-Y responses biased their results. We investigated whether male and female responsiveness in the CML assay is indeed different. Two experiments using PBLs are shown in Fig. 2, panels 1d and 2d; there was no significant sex difference. It is true that another experiment using spleen cells (Fig. 2, panels 1g, 2g) did show a statistically significant difference ($P < 0.05$), with males giving a lower response than females, but quantitatively the difference is so small that it is unlikely to account for the discrepancy. Furthermore, as CBA females rarely make primary anti-H-Y responses⁶ their contribution in the Gorczynski and Steele study¹ can be expected to have been negligible.

(4) Gorczynski and Steele did not test either the injected fathers or their offspring with A/J skin allografts. In our study the experimental fathers were found to be fully non-reactive to skin allografts whereas all their offspring uniformly rejected their grafts.

(5) The CML sensitization and effector assays used by us were not precisely the same as those used by Gorczynski and Steele. They tested only spleen cells and the levels of cytotoxicity they reported¹ were much lower than those obtained in our spleen assays, but it is difficult to make comparisons as their report does not make it clear whether precise cell counts and full titrations were performed on the effector cells generated in culture. It is, however, striking that the low level of cytotoxicity described by them is comparable to the low levels observed by us in some of the experiments in which PBLs were used as responders. The cytotoxicity of both control and experimental mice here tended to be more variable and, we therefore reason, more likely to be sensitive to weak suppressor influences. Our PBL results are nevertheless quite indistinguishable from our spleen results, for neither differentiate between experimental and control offspring.

(6) Whereas all mice in our study were derived from strains bred at St Mary's Hospital Medical School, where the homozygosity of inbred strains is checked from time to time by syngeneic skin transplantation, the mice used by Gorczynski and Steele, although derived from the same foundation stock, were obtained from different breeding colonies. It is conceivable that this introduced a measure of genetic variability that could account for small differences in responsiveness.

(7) We used essentially the same kind of statistical analysis as Gorczynski and Steele. This analysis could fail to reveal a small proportion of non- or hyporesponders in individual experiments, but visual examination of the data suggests that this has not been the case.

It is difficult to account for the discrepancy in the two sets of findings by examining differences in the protocols used. There are available data from several other investigations designed to test the hypothesis put forward by Gorczynski and Steele¹, who have recently extended their observations to another strain combination and claim to have shown the simultaneous and independent inheritance of tolerance to two distinct haplo-

types⁷. R. B. Ashman, C. E. Woodhars and R. V. Blanden (personal communication) tested by CML and skin grafting the progeny of male CBA mice made tolerant at birth by injection of (CBA $\times$ BALB/c)F₁ cells; they did not differ from control progeny although one graft survived on an experimental mouse for a surprisingly long time and this mouse gave a low CML response. McLaren *et al.* (see accompanying letter⁸) examined by CML the reactivity of homozygous progeny of male B6 $\leftrightarrow$ BALB/c tetraparental chimaeric mice bred with both B6 and BALB/c females. The B6 progeny were not hyporesponsive to BALB/c alloantigens, nor were the BALB/c progeny hyporesponsive to B6. J. Craig Gray (personal communication) has been assessing the skin graft survival times in offspring of tolerant male rats, using grafts from the cell donor strain, and here again no hyporesponsiveness has yet been uncovered.

Using a rather different approach, Hašek *et al.*⁹ have been studying CML reactivity of first generation backcross mice of F₁ hybrid H-2 heterozygous fathers. It could be argued that it should follow from the hypothesis under discussion^{1,2} that the natural tolerance of an F₁ father is passed on to his homozygous progeny with respect to their reactivity to the alloantigens of his other parental haplotype. This is evidently not so. Along similar lines, and following the much earlier report of Guttman *et al.*¹⁰, Brent and Rayfield (unpublished data) have been breeding CBA fathers tolerant of A with (CBA $\times$ A)F₁ females to examine the ability of the progeny to reject A skin grafts. In this case positive results would be indicated by an abnormal number of offspring bearing long-surviving A strain grafts compared with control offspring of normal CBA males bred with (CBA $\times$ A)F₁ females. Although the study is not yet complete there are, to date, no differences between the control and experimental groups. Moving away from tolerance to alloantigens, A. Müllbacher, W. Fierz and J. Sheena (personal communication) have bred from CBA mice made tolerant to Bebaru virus by neonatal and subsequent weekly injections of virus inactivated by X-ray irradiation. The offspring of some of the tolerant fathers gave low responses to Bebaru virus, but these were not found in every experiment.

These experiments may be regarded as a test case for the validity of lamarckian inheritance, which relates to the inheritance of acquired adaptations, not to the inheritance of the passively acquired somatic changes. An immunological response is surely the paradigm of physiological adaptations—witness the complete confidence with which an immunologist assumes that a rabbit not yet born will be able to make antibodies against a chemical not yet synthesized. There are technical difficulties in devising critical experiments on the inheritance of antibody formation but these do not apply to specific suppression of immune responsiveness (tolerance): it was therefore enterprising of Gorczynski and Steele to use acquired tolerance as the adaptive response on which to base their examination of lamarckism. Unfortunately, our own findings do not support theirs and confirm the original observations by Billingham *et al.*¹¹ in the identical strain combination. We still believe that all experiments executed hitherto to corroborate the lamarckian interpretation¹² can be faulted.

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Immune reactivity of progeny of tetraparental male mice

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Steele and Gorczynski¹ have recently suggested that inbred male mice rendered tolerant at birth to the alloantigens of an H-2 plus non-H-2 incompatible inbred strain can transmit this tolerance or hyporeactivity, as measured in a primary anti-H-2 cytotoxic T-cell test *in vitro*, to their progeny, born of normally reactive females syngeneic with the males. As a corollary, it might be expected that the progeny of tetraparental males which are tolerant because from earliest fetal development they are chimaeric with respect to all tissues, including haematopoietic cells and germ cells, might in turn be tolerant to the other set of paternal alloantigens. We have now found, on the contrary, that inbred progeny of one component of a tetraparental male showed heightened responsiveness to the other paternal alloantigens.

We examined homozygous progeny of two C57BL/6 ↔ BALB/c male tetraparental chimaeras² with respect to their ability to mount primary anti-H-2 cytotoxic T-cell responses *in vitro* to either C57BL/6 (B6) (H-2^b) or BALB/c (H-2^d) and to a 'third party' strain, CBA (H-2^k). The two chimaeras were mated first with BALB/c females and of the resulting heterozygous (BALB/c × B6)F₁ (agouti) and homozygous BALB/c (white) progeny, the homozygotes were kept and tested. The same tetraparental chimaeras were also bred with B6 females, the agouti (heterozygous B6 × BALB/c) progeny disposed of and the black (homozygous B6) kept and tested. The BALB/c and B6 mothers were mostly used for one litter only. We examined the cytotoxic T-cell responses of a total of 46 offspring from 13 litters, 7 with BALB/c and 6 with B6 mothers. The size of the litters of BALB/c mothers was not statistically different from those of the B6 mothers. There were 26 BALB/c progeny (13 males and 13 females) all of chimaera 1 and 20 B6 progeny (10 males and 10 females) of which 12 were fathered by chimaera 1 and 8 by chimaera 2. Both fathers were coat colour chimaeras but there is evidence only for number 1 being a germ-cell chimaera (see above). Chimaera 2 died before he could be tested for T-cell cytotoxic responses, but chimaera 1 was tested and found to be tolerant to each of the parental strains B6 and

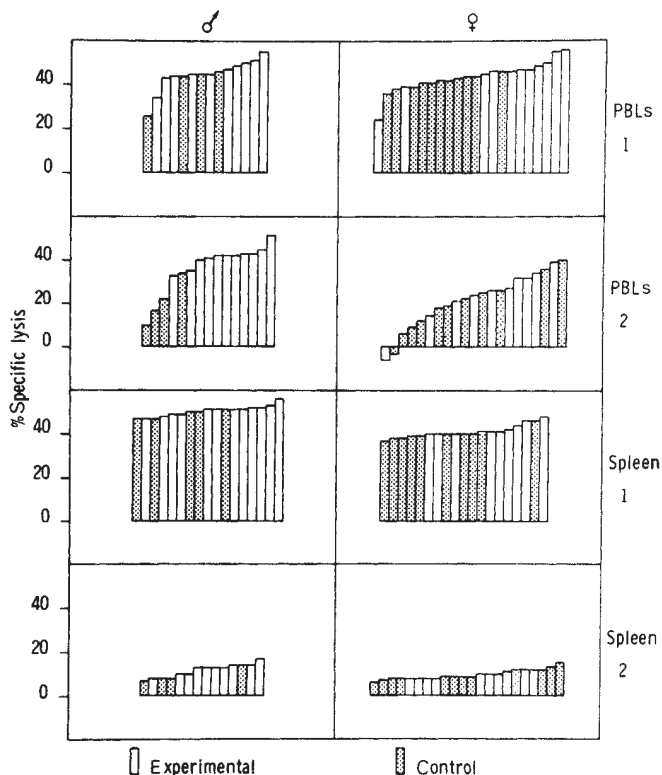


Fig. 1 Anti-C57BL/6 responses of BALB/c progeny of B6 ↔ BALB/c chimaeras and control BALB/c fathers. Conditions of culture. PBLs: two to six 0.2-ml round-bottomed wells were set up with 1×10^5 responders plus 1×10^5 2,000 R irradiated spleen cells per well as antigen, in RPMI with 5% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, penicillin/streptomycin + glutamine. Spleen: 2×4 ml Linbro wells were set up with 1×10^7 responders + 1×10^7 2,000 R irradiated antigen per well in RPMI medium as above. Assay⁴ was on day 5 of culture. Cells were collected from wells, counted, plated and serially double diluted in round-bottomed 0.2-ml microtitre wells, to which were added a constant number of ⁵¹Cr-labelled target cells (24 h concanavalin A blast cells or thioglycollate-induced peritoneal macrophages) to give attacker:target (A:T) ratios starting at ~10:1. They were collected after 3 h incubation. Experimentally determined % specific lysis was subjected to regression analysis. The height of the bars shown is the % specific lysis at A:T 5:1 taken from this curve ($r^2 > 0.9$). The bars representing individual animals are placed in a ranked order.

BALB/c, but reactive to a third party strain, CBA (H-2^k) (Table 1). Previous investigations of chimaeras made in this laboratory have shown that the inability of such mice to make anti-parental cytotoxic responses does not depend on the level of haematopoietic chimaerism. All chimaeras tested were essentially tolerant³.

We used age-matched BALB/c and B6 litters as controls, born of individually identified fathers (four B6 and six BALB/c) and reared in the same environmental conditions as the chimaera progeny. In each experiment either all males or all females were used as responder, antigen and target. Each control and experimental mouse was tested on four different occasions each 2 weeks apart. On two occasions peripheral blood lymphocytes (PBLs) were prepared from blood samples taken by tail bleeding and set up in micromixed lymphocyte culture (MLC)⁴; on the third occasion a sample of spleen was removed for MLC from each mouse under general anaesthesia; finally, the mouse was killed and the remainder of the spleen taken for MLC. During the experiments, a few mice died and the number in each experiment was further reduced by occasional contamination of a culture.

Table 1 Tolerance to parental antigens of a tetraparental chimaera B6 ↔ BALB/c

Responder	Lysis on target		
	B6	CBA	BALB/c
BALB/c (6)	42	44	ND
B6 ↔ BALB/c	4	40	2
B6 (4)	ND	29	21

Tolerance is expressed as % specific lysis at A:T (attacker:target cell ratio) 5:1 in a cytotoxic T-cell response (method as in Fig. 1). Mean values for BALB/c × B6. ND, not done. Numbers of mice tested are given in parentheses.

Table 2 Mean cytotoxic responses of homozygous progeny of two C57BL/6 $\leftrightarrow$ BALB/c made tetraparental chimaeras

		PBLs		Spleen	
		1	2	1	2
B6 anti-BALB/c	E	40.9 (9)	44.2 (9)	26.4 (7)	11.1 (7)
♂	C	39.8 (10)	42.9 (10)	29.5 (10)	9.3 (10)
B6 anti-BALB/c	E	13.9* (8)	19.8 (8)	31.7 (8)	10.0 (5)
♀	C	9.1 (10)	18.8 (10)	31.6 (10)	14.4 (10)
BALB/c anti-B6	E	46.3 (10)	41.5† (11)	50.7 (12)	12.4 (10)
♂	C	39.9 (4)	20.4 (4)	48.9 (5)	9.2 (4)
BALB/c anti-B6	E	45.8 (11)	24.3 (10)	42.5§ (9)	9.8 (10)
♀	C	41.5‡ (11)	19.1 (11)	39.8 (10)	9.8 (12)

Mean cytotoxic responses are expressed as % specific lysis at A:T 5:1 in a cytotoxic T-cell response (method as in Fig. 1). E, experimental animals: progeny of B6 $\leftrightarrow$ BALB/c male chimaeras; C, control animals: age-matched normal animals. Numbers in parentheses are the number of animals tested.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.01$; § $P < 0.05$ (Wilcoxon rank order test).

The mean anti-BALB/c and anti-B6 cytotoxic responses of the various groups are shown in Table 2. Statistical tests (Wilcoxon rank order test) were done on all experiments. The levels of cytotoxicity generated by B6 chimaera male progeny against BALB/c or third party CBA antigen did not differ significantly from those of age- and sex-matched controls. Among the B6 females cells from the chimaera progeny in one test showed significantly greater ($P < 0.05$) cytotoxicity against BALB/c antigen than did the controls, but this result was not confirmed on the three subsequent occasions, and as the ranking data were statistically heterogeneous, they could not be combined. When the responses of the progeny of chimaeras 1 and 2 were analysed independently the same results were obtained. In contrast, when the response of the BALB/c chimaera progeny to B6 antigen was compared with their BALB/c age- and sex-matched controls, a consistent increase in the level of cytotoxicity of the experimental versus control animals was found. Figure 1 shows the responses of all BALB/c progeny tested: for each sex the rank order results from the four occasions proved to be statistically homogeneous, that is, were consistent, and hence could be combined by ranking the summed rank orders. The excess of experimental over control cytotoxicity levels was, though small, statistically significant ($P < 0.01$) for both the male and female progeny independently. The responses of the BALB/c chimaera progeny to the third party control CBA were no different from that of control animals.

These results are in keeping with the findings of Brent *et al.* (see accompanying letter⁵), in that the state of tolerance manifest in male mice is not transmitted to their progeny. In fact, the difference that we observed was in the opposite direction to that predicted from the work of Gorczynski and Steele¹. It occurred whether or not the BALB/c young had F₁ littermates at birth, so is unlikely to be due to sensitization by antigens of litter mates during pregnancy. We are exploring the possibility that it may be mediated via the mother, perhaps as a result of sensitization by antigens present in the ejaculate.

Selective antagonists of benzodiazepines

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Benzodiazepines produce most, if not all, of their numerous effects on the central nervous system (CNS) primarily by increasing the function of those chemical synapses that use γ -amino butyric acid (GABA) as transmitter^{1,2}. This specific enhancing effect on GABAergic synaptic inhibition is initiated by the interaction of benzodiazepines with membrane proteins of certain central neurones, to which drugs of this chemical class bind with high affinity and specificity^{3,4}. The molecular processes triggered by the interaction of these drugs with central benzodiazepine receptors, and which result in facilitation of GABAergic transmission, are still incompletely understood. Theoretically, benzodiazepines could mimic the effect of hypothetical endogenous ligands for the benzodiazepine receptors, although there is no convincing evidence for their existence⁵; *in vitro* studies indicate that benzodiazepines might compete with a modulatory peptide which is present in the supramolecular assembly formed by GABA receptor, chloride ionophore and benzodiazepine receptor and which reduces the affinity of the GABA receptor for its physiological ligand⁶. The mechanisms of action of benzodiazepines at the molecular level are likely to be better understood following our recent discovery of benzodiazepine derivatives, whose unique pharmacological activity is to prevent or abolish in a highly selective manner at the receptor level all the characteristic centrally mediated effects of active benzodiazepines. Here, we describe the main properties of a representative of this novel class of specific benzodiazepine antagonists.

Working on a series of imidazodiazepines, we found compounds which were potent inhibitors of the specific high-affinity binding of ³H-diazepam to brain synaptosomal fractions, but which failed to produce any of the behavioural and neurological effects typical of benzodiazepines. Detailed analysis revealed that these compounds selectively and potentially inhibit the central effects of classic benzodiazepines by acting as their antagonists at benzodiazepine receptors. From a large series of compounds we have selected Ro 15-1788 (Fig. 1) for clinical trials.

Ro 15-1788 is even less toxic than most of the classic benzodiazepines; the following approximate acute LD₅₀ values were determined: 4,000 mg per kg intraperitoneally (i.p.) and 4,300 mg per kg orally (p.o.) in mice, 1,360 mg per kg i.p. and 6,000 mg per kg p.o. in rats. In mice, rats, cats, dogs and squirrel monkeys the compound up to subtoxic doses produces none of the characteristic effects of benzodiazepine drugs, such as sedative, anticonvulsant, muscle relaxant and anticonflict effects. Ro 15-1788 is not a convulsant by itself, it fails to sensitize animals to chemical convulsants such as leptazol and lacks overt central stimulant activity in all species studied (L.P. *et al.*, in preparation).

Specific binding of ³H-diazepam to crude synaptosomal fractions of rat cerebral cortex⁴ was inhibited competitively by Ro 15-1788 (Fig. 2) with an IC₅₀ value of 2 nmol l⁻¹. This is about one quarter the value for diazepam in the same experimental conditions. Ro 15-1788 also dose dependently inhibited the binding in the brain of ³H-flunitrazepam injected intravenously (i.v.) in mice (H.M. *et al.*, in preparation): tested 15 min after

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p.o. administration, Ro 15-1788 was about half as potent as diazepam (ED_{50} values 4.0 and 1.7 mg per kg, respectively).

Three test procedures were adapted routinely to screen for potential benzodiazepine antagonism: the anti-leptazol test⁷, the horizontal wire test⁸ in mice and the antagonism of flunitrazepam-induced pre-anaesthesia in squirrel monkeys. In the first test, a supramaximal i.p. dose of leptazol (120 mg per kg) produced tonic seizures in all animals. Diazepam 5 mg per kg i.p. (twice the calculated ED_{90}) fully protected mice from tonic seizures when given 1 h before leptazol. Ro 15-1788 was administered p.o. or parenterally at various doses and varying times after diazepam and the number of animals developing tonic seizures in response to leptazol were counted. When given p.o. 15 min before leptazol (and 45 min after diazepam), Ro 15-1788 antagonized the anticonvulsant effect of diazepam with an ED_{50} of 2.8 mg per kg, but did not reduce the anticonvulsant action of phenobarbitone in the same test procedure.

In the horizontal wire test⁸, central depressant drugs dose dependently impair the ability of mice hung on a wire by their forepaws to heave themselves on to the wire with their hindlegs. Diazepam (3 mg per kg i.p.) reduced the number of mice performing on the horizontal wire by 70–100%. Ro 15-1788 given before, simultaneously with or after diazepam dose dependently attenuated and blocked the depressant effect of diazepam and of other benzodiazepines; when given 15 min after 3 mg per kg diazepam i.p. and tested over 30 min, the ED_{50} value for Ro 15-1788 was 0.2 mg per kg p.o. Ro 15-1788 by itself did not affect the performance of mice and up to very high doses (300 mg per kg) was unable to antagonize the depressant effect of phenobarbitone, meprobamate, ethanol, L-cycloserine (a GABA transaminase inhibitor) and muscimol. However, it antagonized the performance-depressant effect of zopiclone⁹, a non-benzodiazepine with diazepam-like activity and moderate potency as an inhibitor of specific ³H-diazepam binding to brain synaptosomes. A pre-anaesthetic state (loss of righting reflex, ataxia, sleep-like posture) was induced in squirrel monkeys by 3 mg per kg flunitrazepam i.v. When Ro 15-1788 was administered by stomach tube in doses between 10 and 100 mg per kg at the peak of flunitrazepam action, the monkeys stood up spontaneously within minutes and returned to normal behaviour for 2–4 h.

Increase of food-rewarded behavioural responses that are depressed by a punishment contingency is considered to be the most relevant available indication from animal experiments for anxiolytic activity in man. In the procedure used in the present experiments, rats were trained to press a lever for food reward; during 'conflict sessions', in which each lever-press was both rewarded with a food pellet and punished with an electric foot shock, the number of lever presses was markedly reduced. Diazepam (5 mg per kg p.o.) consistently increased the number of punished responses. Ro 15-1788 at 10 mg per kg p.o. prevented the antipunishment effect of diazepam.

Electrophysiological studies revealed the following effects of Ro 15-1788 (P.P. *et al.*, in preparation). In non-anaesthetized acute spinal cats the compound did not itself affect segmental dorsal root potentials and reflexes, polysynaptic ventral root reflexes and the spontaneous discharge rate of γ -motoneurons (for methods see ref. 10). The very potent benzodiazepine, Ro 11-3128 (3-methylclonazepam), at 0.1 mg per kg i.v., markedly

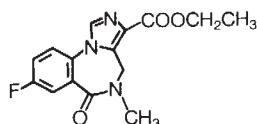


Fig. 1 Structural formula of Ro 15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5a][1,4]benzodiazepine-3-carboxylate).

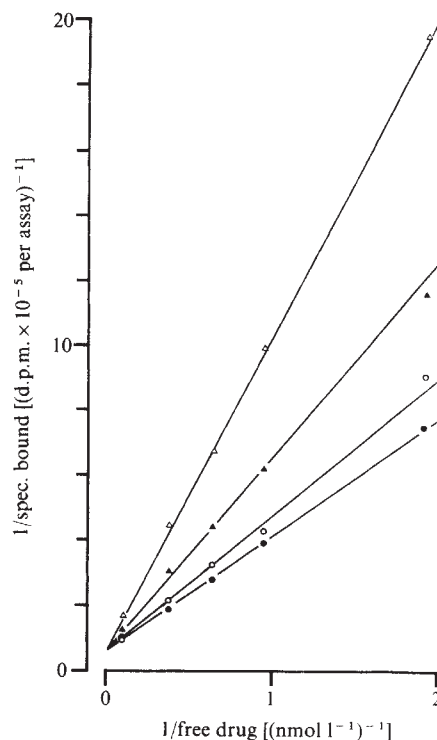


Fig. 2 Inhibition of specific binding of ³H-diazepam (0.5–10 nmol l⁻¹) in rat cerebral cortex homogenate by Ro 15-1788. ○, 0.5 nmol l⁻¹; ▲, 2 nmol l⁻¹; △, 5 nmol l⁻¹; ●, no Ro 15-1788.

augmented the dorsal root potential (index of GABA-mediated primary afferent depolarization) and depressed polysynaptic ventral root reflexes and γ -motoneurone activity. These effects were dose dependently reversed by i.v. injections of Ro 15-1788. In non-anaesthetized 'encéphale isolé' rats, i.v. injections of the short-acting imidazobenzodiazepine, midazolam⁷, produced a dose-dependent decrease in the spontaneous multi-unit activity in the substantia nigra pars compacta and in the nucleus raphé dorsalis; a 50% decrease was achieved between 326 and 750 μ g per kg. Ro 15-1788 at 3 mg per kg i.v. reversed the effect of the highest dose of midazolam on the firing rate of these supposed dopamine and serotonin neurones. In the same rats essentially the same antagonism between midazolam and Ro 15-1788 was found on hippocampal neurones.

Ro 15-1788 antagonized characteristic biochemical changes induced in the brain by benzodiazepines (H.M. *et al.*, in preparation). These drugs consistently attenuate the neuroleptic-induced increase of homovanillic acid (HVA) in the rat brain (an index of dopamine turnover) by enhancing a GABAergic inhibitory influence on dopamine neurones¹¹. Ro 15-1788 dose dependently reversed the reduction by diazepam of the chlorpromazine-induced increase of HVA. Benzodiazepines and sedative drugs of diverse chemical classes decrease the cyclic 3',5'-guanosine monophosphate (GMP) content of the cerebellum in rats^{12,13}. Ro 15-1788 by itself did not affect the cerebellar cyclic GMP level, but dose dependently prevented (when given before) and attenuated or abolished (when given afterwards) the cyclic GMP reduction induced by diazepam. The reduction of cerebellar cyclic GMP by barbiturates, ethanol, meprobamate, muscimol and neuroleptics was unaffected by Ro 15-1788.

The antagonistic activity of Ro 15-1788 is not only selective for active benzodiazepines as distinct from non-benzodiazepine depressants but is also selective for benzodiazepine effects mediated by the so-called central type of benzodiazepine receptor as distinct from effects produced through peripheral types of receptor or through nonspecific interactions with

biological systems. For example, the antischistosomal effect of Ro 11-3128 (refs 14, 15) was not inhibited by Ro 15-1788 *in vitro* and *in vivo* (H. R. Stohler, personal communication); the antischistosomal activity of some 7-nitro-substituted benzodiazepine derivatives is mediated by a low-affinity binding site in the epidermis of schistosomes with a different ligand specificity from that of the central type of benzodiazepine receptor¹⁶. Ro 15-1788 also failed to antagonize the depression by benzodiazepines in high concentrations (10^{-5} – 10^{-3} mol l⁻¹) of contractile responses of the guinea pig ileum to transmural electrical stimulation, an effect which is probably due to a nonspecific membrane stabilizing action.

These results characterize Ro 15-1788 as a representative of a novel class of drugs that act as specific antagonists of CNS effects of active benzodiazepines as well as of non-benzodiazepines with affinity for the central type of benzodiazepine receptors. The discovery of such antagonists is analogous to that of opiate antagonists and has considerable practical and scientific implications. They should be useful as antidotes for intoxications involving overdoses of benzodiazepines. As non-toxic agents, they should be useful for abolishing or reducing central effects of benzodiazepines given for surgical or diagnostic interventions whenever an immediate reversal of benzodiazepine effects is required. Previous attempts to counteract benzodiazepine effects by cholinesterase inhibitors^{17,18} or naloxone^{19,20} have yielded erratic results. Diagnostically, benzodiazepine antagonists should easily unmask the presence of active amounts of benzodiazepines in the CNS. Ro 15-1788 will be used to prevent the undesired central effects of Ro 11-3128 (3-methylclonazepam), which has proved effective as a one-shot treatment of schistosomiasis. Indeed, pilot studies in human volunteers have demonstrated a good tolerance and lack of intrinsic pharmacological effects of Ro 15-1788 as well as its ability to antagonize sedation induced by Ro 11-3128 (A. Darragh, personal communication). As research tools, Ro 15-1788 and other specific benzodiazepine antagonists will be extremely useful in defining the structural properties required for molecules to be recognized and bound by benzodiazepine receptors and to induce or prevent those conformational changes in the receptor protein that result in the typical enhancement of GABAergic transmission. Benzodiazepine antagonists should also be powerful tools in studying the possible existence of endogenous ligands for benzodiazepine receptors. Our screening for benzodiazepine antagonistic activity resulted in the identification of many compounds possessing either pure antagonistic or antagonistic properties combined with weak agonistic activity and revealed interesting and unexpected structure-activity relationships.

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Purified EGF receptor-kinase interacts specifically with antibodies to Rous sarcoma virus transforming protein

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Transformation by several RNA tumour viruses seems to be mediated by virally coded protein kinases which specifically phosphorylate tyrosine^{1–7}. A tyrosine-specific protein kinase also seems to be involved in the mitogenic action of epidermal growth factor (EGF)⁸. This EGF-stimulated kinase activity is closely associated with the EGF receptor, with which it co-purifies during EGF-affinity chromatography⁹. Because both the virus- and EGF-stimulated tyrosine kinases may be involved in stimulation of cell growth, and because the viral kinases may be antigenically related to normal cell proteins^{5,10–12}, we examined the interaction of antibodies to viral tyrosine kinases with the affinity-purified EGF receptor-kinase preparation. We report here that the receptor-kinase specifically phosphorylates antibodies directed against the transforming protein kinase pp60^{src} of Rous sarcoma virus. However, none of these antibodies, including those which cross-react with the normal cellular homologue of pp60^{src} (pp60^{src}), precipitate the receptor-kinase. These results suggest that the EGF receptor-kinase is related to, but probably not identical with, pp60^{src}.

Antisera from rabbits bearing tumours induced by Rous sarcoma virus (TBR sera) not only precipitate pp60^{src} (ref. 13), but also have the unusual property of acting as a specific substrate for the protein kinase activity of pp60^{src}. In this reaction, pp60^{src} catalyses the phosphorylation of the IgG heavy chain^{14,15}, but does not phosphorylate normal rabbit IgG^{16,17}. TBR IgGs are not substrates for protein kinases in general¹¹. Using TBR sera obtained from several laboratories, we examined the ability of the EGF receptor-kinase to phosphorylate specifically the IgG heavy chain of TBR sera.

Affinity-purified EGF receptor-kinase, prepared from membranes of A431 cells⁹, was incubated with various antisera in the presence of EGF, Mn²⁺ and [γ -³²P]ATP and subjected to SDS-gel electrophoresis and autoradiography (Fig. 1). As previously reported, in the absence of added serum the receptor-kinase catalyses the phosphorylation of two bands with apparent molecular weights (MWs) of 150,000 and 170,000 (Fig. 1, lane a)—this doublet is thought to be the EGF receptor itself⁹. When TBR serum is included in the reaction mixture, a band co-migrating with the IgG heavy chain becomes phosphorylated (Fig. 1, lanes b–f). This phosphorylation seems to be more pronounced when TBR sera that cross-react with the normal cellular homologue (pp60^{src}) of pp60^{src} are used (Fig. 1, lanes d–f). Normal rabbit sera are not phosphorylated or are phosphorylated only to a small extent (Fig. 1, lanes g, h). We tested 30 antisera to various proteins and viruses in this assay, and, with the exception of TBR sera, none was phosphorylated by the EGF receptor-kinase. These controls included antisera which precipitate the tyrosine kinases associated with Abelson murine leukaemia virus¹, feline sarcoma virus⁵ and polyoma virus¹⁸, as well as an antiserum to A431 cell membranes that precipitates the receptor-kinase (Fig. 1, lane i)¹⁹. Thus, the EGF receptor-kinase interacts specifically with antisera that precipitate pp60^{src}.

The observed phosphorylation of TBR IgG was not due to endogenous protein kinase activity in the TBR sera or to an EGF-unrelated cellular kinase in the purified receptor prep-

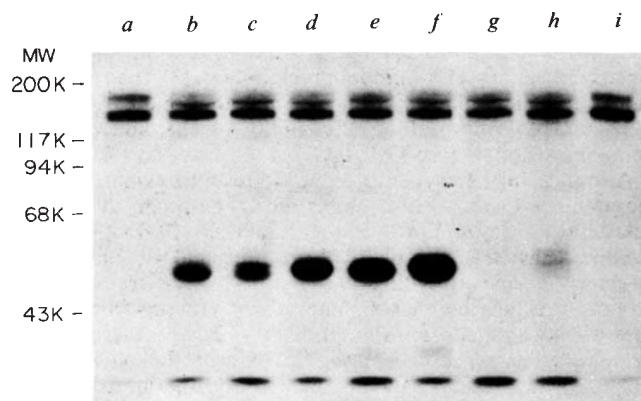


Fig. 1 Analysis by SDS-gel electrophoresis and autoradiography of EGF receptor-kinase-catalysed phosphorylation of rabbit anti-tumour sera. Antisera from rabbits bearing tumours induced by Rous sarcoma virus (TBR sera)¹³ were able to precipitate and act as a substrate for the transforming protein kinase coded for by Rous sarcoma virus (pp60^{src})^{14,15}. The antisera shown in lanes *d-f* can also precipitate, and be phosphorylated by, the homologous cellular pp60^{src} (ref. 11). Rabbit antiserum raised against A431 human epidermal carcinoma cell membranes was prepared and purified as described elsewhere¹⁹; this antiserum precipitates the purified EGF receptor with its associated tyrosine-specific⁸ protein kinase activity (EGF receptor-kinase). The EGF receptor-kinase was purified from Triton/glycerol-solubilized A431 cell membranes by adsorption to EGF-Affigel and elution with ethanolamine as described elsewhere⁹. The receptor-kinase preparation used in these experiments contained ~90 µg ml⁻¹ of protein in 1% Triton X-100, 10% glycerol, 5 mM ethanolamine, pH 7. 15 µl of receptor-kinase was activated⁹ by incubation for 10 min at 25 °C with 15 µl EGF (100 ng, in 0.05% bovine serum albumin (BSA)) and 15 µl of 4 mM MnCl₂ in 60 mM HEPES, pH 7.4 (HEPES/Mn²⁺). The mixture was then chilled on ice for 10 min and incubated with 3 µl of the indicated antiserum for 30 min at 0 °C. 15 µl [γ -³²P]ATP (2 µCi, 60 µM) was then added, and after 10 min at 0 °C the phosphorylation reaction was terminated by addition of SDS sample buffer to the final concentrations described by Laemmli²¹. Samples were heated for 5 min in a boiling water bath and electrophoresed through a 3% polyacrylamide stacking gel and a 7.5% separating gel using Laemmli's buffer system²¹. ³²P-labelled proteins were visualized by autoradiography on DuPont Cronex film, while molecular weight markers (BioRad) and the IgG heavy chain were located by Coomassie blue staining. Markers were myosin, MW 200,000 (200 K); β -galactosidase, MW 117 K; phosphorylase *b*, MW 94 K; BSA, MW 68 K, and ovalbumin, MW 43 K. Serum additions were as follows: *a*, saline control; *b-f*, TBR sera; *g*, *h*, normal rabbit sera; *i*, rabbit anti-A431 cell membrane IgG (25 µg).

aration (Fig. 2) because no phosphorylation of IgG was observed in any of the TBR sera in the absence of added receptor-kinase (Fig. 2, lane *a*), and phosphorylation of the IgG heavy chain was stimulated by EGF (Fig. 2, lanes *b*, *c*).

Surprisingly, despite the specific interaction of the EGF receptor-kinase with TBR IgG, none of the TBR sera examined was able to precipitate the receptor-kinase, including antisera known to precipitate pp60^{src} from uninfected cells (Fig. 3). The receptor doublet and the IgG heavy chain were labelled with ³²P by incubating receptor-kinase with TBR serum in the presence of EGF, [γ -³²P]ATP and Mn²⁺ (Fig. 3, lane *a*). This prelabelled mixture was then subjected to immunoprecipitation with protein A-Sepharose, and the supernatant (Fig. 3, lane *b*) and washed pellet (Fig. 3, lane *c*) were examined by SDS-gel electrophoresis and autoradiography. The phosphorylated IgG is precipitated but the receptor remains in the supernatant. However, as it is possible that the receptor and kinase are dissociable, this experiment was not adequate to determine

whether the receptor-associated kinase activity had remained in the supernatant or had been precipitated with the TBR IgG. Therefore, unlabelled EGF receptor-kinase, pre-activated with EGF, was incubated with unlabelled TBR serum, immunoprecipitated with protein A-Sepharose, and the supernatant and washed pellet were assayed for protein kinase activity in the presence of [γ -³²P]ATP and Mn²⁺. All phosphorylating activity was found in the supernatant, where the receptor doublet became phosphorylated (Fig. 3, lane *d*), whereas the IgG-containing precipitate contained no detectable kinase activity (Fig. 3, lane *e*). Thus, neither the EGF receptor nor its associated protein kinase activity is precipitated by TBR serum. The antisera to other viral tyrosine kinases (see above) also failed to precipitate the EGF receptor (not shown). As a positive control, it was confirmed that this TBR serum could precipitate IgG kinase activity from a crude detergent extract of A431 cells (Fig. 3, lane *g*). Thus, although A431 cells seem to contain pp60^{src} that precipitates with and phosphorylates TBR serum, this kinase is not detected in the purified receptor preparation. Furthermore, in these conditions both the receptor and its associated kinase activity could be immunoprecipitated using antiserum directed against the A431 cell membranes from which the receptor-kinase was purified (Fig. 3, lane *f*). In this experiment, unlabelled receptor-kinase, precipitated with the anti-membrane serum, became labelled in the immunoprecipitate on addition of [γ -³²P]ATP and Mn²⁺; the antibody was not labelled.

Because phosphorylation of IgG catalysed by pp60^{src} and phosphorylation of the 150,000–170,000 MW EGF receptor doublet by the receptor-kinase are known to occur on tyrosine residues^{2,8}, it was interesting to determine whether the IgG phosphorylation catalysed by the receptor-kinase was also specific for tyrosine (Fig. 4). TBR IgG was phosphorylated in the presence of the receptor-kinase and [γ -³²P]ATP, and precipitated with protein A-Sepharose; the precipitated material was eluted from the washed beads by heating in SDS, and SDS-gel electrophoresis and autoradiography of an aliquot of this eluate were used to confirm that the only major phosphorylated component was the IgG heavy chain (Fig. 4*a*). The remainder of the eluted material was subjected to partial acid hydrolysis and paper electrophoresis at pH 3.5 in the presence of internal phosphoamino acid standards⁸. With or without EGF,

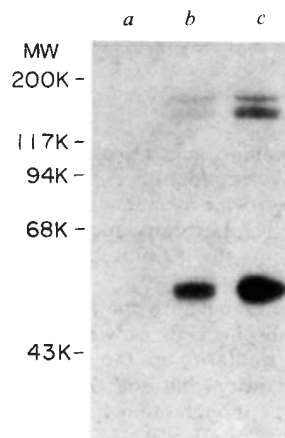


Fig. 2 Effect of EGF on receptor-kinase-catalysed phosphorylation of TBR serum. Phosphorylation reactions, electrophoresis and autoradiography were carried out as described in the legend to Fig. 1, except that buffer blanks were substituted for EGF or for EGF receptor-kinase as indicated. *a*, Minus receptor-kinase, +EGF, +TBR serum; *b*, plus receptor-kinase, -EGF, +TBR serum; *c*, plus receptor-kinase, +EGF, +TBR serum. Similar results (not shown) were obtained with the other TBR sera described in Fig. 1 legend.

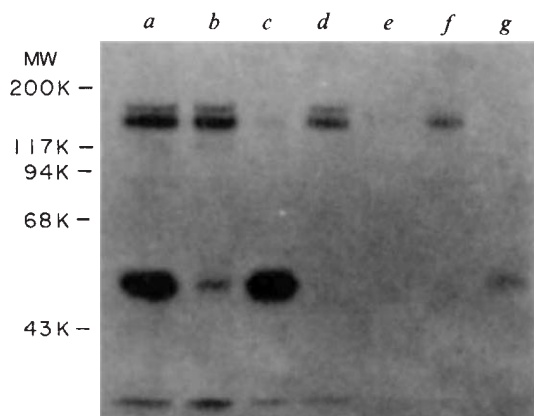


Fig. 3 SDS-gel electrophoresis and autoradiography showing non-precipitation of EGF receptor-kinase by TBR serum. *a-c*, Mixtures of EGF receptor-kinase and TBR serum (known to cross-react with pp60^{sarc}) were phosphorylated as described in Fig. 1 legend, except that the final reaction volume was increased to 83 μ l. *a*, This phosphorylated mixture was subjected to SDS-gel electrophoresis without further treatment; *b, c*, IgG in a replicate phosphorylated mixture was adsorbed to 40 μ l of packed protein A-Sepharose in 20 mM HEPES, pH 7.4 by incubation for 30 min at 0 °C with resuspension every 5 min. The sample was then spun for 2 min at 8,000g in an Eppendorf minifuge, and 60 μ l of the supernatant prepared for electrophoresis (lane *b*). The protein A-Sepharose beads were washed five times with 1 ml of 20 mM HEPES, pH 7.4. The bound IgG was eluted by heating for 5 min at 100 °C in 150 μ l of SDS sample buffer, and, after removal of the protein A-Sepharose beads by centrifugation, was subjected to SDS-gel electrophoresis (lane *c*). *d, e*, Unlabelled receptor-kinase was activated with EGF and incubated with TBR serum as in *a-c*. Following incubation, IgG was precipitated with protein A-Sepharose as described above for lanes *b* and *c*; 60 μ l of the supernatant was incubated for 10 min at 0 °C with 20 μ l each HEPES/Mn²⁺ and [γ -³²P]ATP (2.0 μ Ci, 60 μ M) and processed for electrophoresis (lane *d*). The protein A-Sepharose precipitate was washed five times with 1 ml of 20 mM HEPES, pH 7.4, and incubated for 10 min at 0 °C with 20 μ l each HEPES/Mn²⁺ and [γ -³²P]ATP. IgG bound to the protein A-Sepharose was eluted as for *c* and electrophoresed (lane *e*). *f, g*, Positive controls for immunoprecipitation: *f*, as lane *e*, except that rabbit IgG raised against A431 cell membranes was used in place of TBR serum; *g*, a crude 1% Triton/10% glycerol extract of unlabelled A431 cells was incubated with cross-reactive TBR serum and immunoprecipitated. Precipitation with protein A-Sepharose and phosphorylation of the precipitate with [γ -³²P]ATP was as for samples *e* and *f*.

sufficiently antigenically related to pp60^{sarc} specifically to bind and phosphorylate anti-pp60^{sarc} IgG, but insufficiently related to bind tightly enough for immunoprecipitation. (3) The receptor-kinase may be pp60^{sarc}, but the interaction of pp60^{sarc} with the EGF receptor may inhibit the ability of the kinase to be immunoprecipitated by TBR IgG.

Although further purification of the receptor-kinase will be required to distinguish unambiguously between the above possibilities, we find it intriguing that an EGF-stimulated enzyme which seems to be involved in normal growth control interacts specifically with antibodies to a viral transforming protein. It is possible that the normal cell proteins from which viral transforming proteins are thought to have evolved²⁰ may, like the EGF receptor-kinase, be part of the normal cell machinery involved in growth regulation. Furthermore, as EGF is not tumorigenic, we believe that study of the means by which different tyrosine kinases stimulate growth may elucidate the cellular controls that distinguish malignant from non-malignant growth.

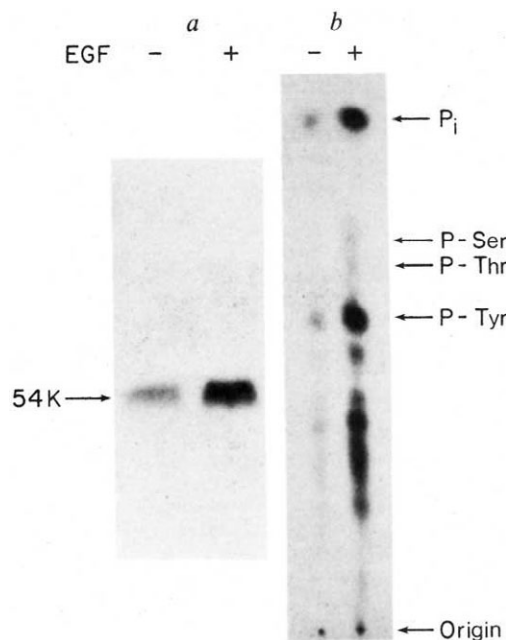


Fig. 4 Identification of phosphotyrosine in TBR IgG phosphorylated by the EGF receptor-kinase in the absence/presence of EGF. 40 μ l of the receptor-kinase preparation was mixed with 40 μ l of HEPES/Mn²⁺ and 40 μ l of either 0.05% BSA without EGF, or 0.05% BSA containing 270 ng EGF, and was incubated for 10 min at 25 °C. The mixture was chilled on ice for 10 min and incubated for 1.5 h at 0 °C with 6 μ l of TBR serum. 4.5 μ l of [γ -³²P]ATP (35 μ Ci, 436 μ M) was then added, and incubation on ice was continued for 10 min. 40 μ l of protein A-Sepharose in 20 mM HEPES, pH 7.4 was then added, and the suspension incubated for 30 min on ice, with resuspension every 5 min. The protein A-Sepharose was washed five times with 1 ml of 20 mM HEPES, pH 7.4 by spinning for 2 min at 8,000g in an Eppendorf minifuge, and the bound IgG was eluted by adding 600 μ l of 2% SDS in 20 mM HEPES, pH 7.4 and heating for 5 min in a boiling water bath. Aliquots (50 μ l) of each sample were examined by SDS-gel electrophoresis and autoradiography (*a*). The remainders of the samples were transferred to glass centrifuge tubes on ice and precipitated with 20% trichloroacetic acid (TCA) in the presence of 100 μ g BSA and 10 mM sodium pyrophosphate. Precipitates were washed twice with 20% TCA/10 mM sodium pyrophosphate and once each with ethanol and ether. Samples were air dried, hydrolysed under vacuum in 6 M HCl for 2.5 h at 100 °C, and processed for paper electrophoresis at pH 3.5, as described elsewhere⁸. Results of paper electrophoresis and autoradiography are shown in *b*. Internal phosphoamino acid standards⁸ were located by staining with ninhydrin.

the major phosphoamino acid released by hydrolysis of the phosphorylated IgG was identified as phosphotyrosine; the amount of phosphotyrosine formed was greater in the presence of the hormone. Thus, phosphorylation of TBR IgG by either pp60^{sarc} (ref. 2) or by the EGF receptor-kinase occurs on tyrosine.

The previous findings that both pp60^{sarc} (refs 2, 4) and the EGF receptor-kinase⁸ phosphorylate only tyrosine residues suggested some possible similarity in the active sites of the two enzymes. Our observation that both kinases specifically phosphorylate TBR IgG, although other IgGs contain tyrosine residues, suggests further similarity. However, there are several interpretations of the degree of this similarity: (1) TBR IgG, although not substrates for cyclic AMP-dependent protein kinases¹¹, may contain sequences that make them efficient substrates for a variety of related tyrosine-specific protein kinases. In this regard, it is interesting that the tyrosine kinase activities associated with the transforming proteins of feline sarcoma virus, Abelson murine leukaemia virus, polyoma virus, and the avian sarcoma viruses Y73 and PRC II are able specifically to phosphorylate TBR IgG (K. Beemon and T. Hunter, personal communication). (2) The EGF receptor-kinase may be

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Anti-pp60^{src} antibodies are substrates for EGF-stimulated protein kinase

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Epidermal growth factor (EGF) stimulates phosphorylation of its own receptor at a tyrosine residue¹. Similarly, the viral gene product pp60^{src}, which is responsible for cellular transformation by avian sarcoma virus (ASV), phosphorylates itself and immunoglobulin directed against pp60^{src} at tyrosine residues^{2,3}. This unusual site of phosphorylation catalysed by two membrane-associated protein kinases^{4–7} involved in growth control prompted us to study the immunological relatedness of the EGF-stimulated protein kinase and the pp60^{src}. Using anti-pp60^{src} antisera, we attempted to immunoprecipitate the EGF-stimulated protein kinase solubilized from plasma membranes. We report here that neither the EGF-stimulated kinase nor the EGF receptor were immunoprecipitable by anti-pp60^{src} sera. However, anti-pp60^{src} IgG served as a specific substrate for the EGF-stimulated kinase, suggesting a close similarity between the EGF-stimulated kinase and pp60^{src}.

We used the plasma membranes of A431 human carcinoma cells because of their unusually high density of EGF receptors⁸. Detergent-solubilized plasma membranes prepared from these cells retain EGF-stimulated phosphorylation of the EGF receptor protein⁹ of molecular weight (MW) ~170,000. Treatment of the solubilized membranes with antisera raised in ASV tumour-bearing rabbits¹⁰ was followed by removal of the immune complexes with protein A containing *Staphylococcus aureus* (Pansorbin, Calbiochem). Phosphorylation of immune, but not control, IgG heavy chains could be demonstrated in the resultant precipitate. Normal or non-ASV-transformed vertebrate cells contain *src* gene sequences and a cell protein

(pp60^{src}) that is closely related to the viral pp60^{src} (refs 11–14). Our ability to precipitate specifically a rabbit IgG heavy chain kinase with anti-pp60^{src} sera suggested that the A431 plasma membrane contained pp60^{src}.

Using this immunoprecipitation approach, we determined the effect of removal of pp60^{src} activity on EGF-stimulated phosphorylation of the EGF receptor. The membranes were solubilized using a procedure that optimized the recovery of pp60^{src} activity in the immunoprecipitates and the solubilized A431 membranes were then titrated with antiserum to determine the amount required to remove the maximum quantity of pp60^{src} activity made available by membrane solubilization. As determined by this titration, doubling of the amount of antiserum did not increase the amount of pp60^{src} activity precipitated. However, another incubation with additional antiserum in the immune adsorbed supernatant detected some residual pp60^{src} activity (Fig. 1, lane 8), which never exceeded 25% of the total immunoprecipitable activity. A third immune precipitation failed to detect any residual pp60^{src} activity. When immunoprecipitates were formed from solubilized plasma membranes

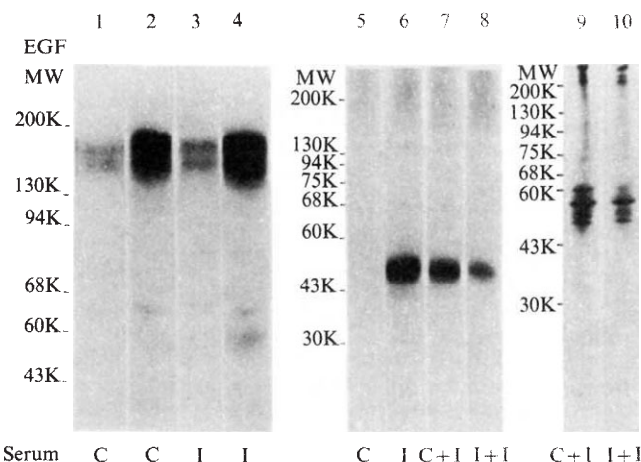


Fig. 1 Effect of immunoprecipitation of pp60^{src} activity on EGF-stimulated phosphorylation of the EGF receptor in solubilized A431 cell membranes. Purified A431 plasma membranes¹⁷ were solubilized in buffer containing 1% Triton X-100, 10% glycerol, and 20 mM HEPES, pH 7.4 (ref. 9). Control (lanes 1, 2) and anti-pp60^{src} (lanes 3, 4) sera at a final dilution of 1/20 were incubated with 400 µg ml⁻¹ of solubilized A431 membrane for 3 h in 20 mM HEPES, pH 7.4, 1 mM MnCl₂, and 0.1 mg ml⁻¹ bovine serum albumin (BSA). Control (lane 5) and immune (lane 6) IgG were removed from the mixture by precipitation with Pansorbin for quantitation of pp60^{src} activity and the remaining supernatants divided into two aliquots: one was treated with (lanes 2, 4) or without (lanes 1, 3) 0.3 µM EGF (ref. 9) followed by the addition of 15 µM [³²P]ATP (1.25 µCi per 60 µl assay). After incubation for 2 min at 0 °C, the reaction was stopped by the addition of Laemmli sample buffer¹⁸ and boiling for 3 min, followed by electrophoresis on 7.5% polyacrylamide gels. Phosphorylated proteins were visualized by autoradiography. To quantify residual pp60^{src} present in control and immune supernatants, second aliquots of supernatant were again treated with anti-pp60^{src} serum and Pansorbin. These second immune precipitates derived from control (lane 7) or immune (lane 8) serum-treated supernatants were assayed for pp60^{src} activity. The Pansorbin-bound immune precipitates were washed three times by centrifugation in NP40 buffer¹⁹, once in phosphate-buffered saline, and resuspended in 80 µl of a buffer containing 20 mM sodium phosphate (pH 7.2), 5 mM MgCl₂ and 15 nM [³²P]ATP (3,000 Ci mmol⁻¹, 2 µCi per assay). After incubation for 20 min at 30 °C, the reaction was stopped by the addition of RIPA buffer²⁰, and phosphorylated proteins were separated on 10% polyacrylamide gels. The peptides present in the immunoprecipitates were analysed (lanes 9, 10). A431 cells were metabolically labelled with ³⁵S-methionine¹⁴, the plasma membranes isolated and solubilized and immunoprecipitations performed. The Pansorbin-bound immunoprecipitates from solubilized membranes that had been pre-adsorbed with control (lane 9) or immune (lane 10) serum were washed extensively before separation on 10% polyacrylamide gels. Molecular weight standards were: myosin, MW 200,000 (200 K); β-galactosidase, 130 K; phosphorylase b, 94 K; transferrin, 75 K; BSA, 68 K; catalase, 60 K; ovalbumin 43 K, and carbonic anhydrase, 30 K. C designates treatment with control serum, I with anti-pp60^{src} serum and C+I or I+I, sequential treatment with these sera.

Table 1 EGF-stimulated phosphorylation of tyrosine residues in the EGF receptor and in anti-pp60^{src} IgG

	³² P incorporation into tyrosine residues (c.p.m.) EGF receptor (MW ~ 170,000)	IgG heavy chain (MW = 55,000)
- EGF	156	147
+ EGF	349	513

Phosphorylation reaction mixtures containing solubilized A431 membranes and anti-pp60^{src} serum with or without 0.3 μ M EGF were incubated as described in the legends to Figs 1 and 2. After electrophoresis, the gels were dried and the phosphorylated EGF receptor and IgG heavy chain located by autoradiography using appropriate alignment markers. The regions of the dried gel containing the phosphoproteins were cut out and placed in dialysis bags after removal of the paper backing. Buffer containing freshly prepared 10 mM NH_4HCO_3 , 0.1% SDS and 5% 2-mercaptoethanol was placed in the dialysis bag to swell the gel. The dialysis bags were placed in the same buffer between two platinum electrodes and a 5–10 V cm^{-1} field applied for 8 h; the field was reversed for 15 s before removal of the contents. This solution was filtered through glass wool and 50 μ g of bovine γ -globulin carrier added before trichloroacetic acid (TCA) precipitation. The protein precipitate was washed in ethanol and ether and hydrolysed for 2 h in 6 M HCl at 110 °C. Phosphoamino acids were separated by electrophoresis on cellulose thin layer plates with the buffer (pH 3.5) described by Hunter and Sefton². ³²P-labelled amino acids were located by autoradiography and identified by alignment with the corresponding ninhydrin-stained phosphoamino acid standards. The area of the thin layer plate corresponding to phosphotyrosine was scraped and its ³²P content determined by scintillation counting.

prepared from ³⁵S-methionine-labelled A431 cells, several peptides were precipitated nonspecifically (data not shown). However, if immunoprecipitates were formed from solubilized membranes pre-adsorbed with control serum, a peptide of MW 60,000 was evident. Pre-adsorption with anti-pp60^{src} serum markedly and specifically diminished the amount of the 60,000-MW peptide in the immunoprecipitate, approximately in proportion to the removal of anti-pp60^{src} IgG kinase activity (Fig. 1, lanes 9, 10). Despite specific removal of >75% of the pp60^{src} activity and the associated 60,000-MW peptide from the solubilized membrane, EGF-stimulated phosphorylation of the ~170,000-MW receptor protein was unaffected (Fig. 1, lanes 1–4). Furthermore, analysis of the products of phosphorylation of the immunoprecipitate never revealed the ~170,000-MW receptor (Fig. 1, lanes 6–8) nor could EGF receptor phosphorylated before immunoprecipitation be detected in the immunoprecipitates (See Fig. 3b). Thus, neither the EGF receptor nor the EGF-stimulated protein kinase was removed by immunoprecipitation. These data suggested that either the pp60^{src} was immunologically unrelated to the EGF-stimulated kinase or that it was unavailable for immune adsorption.

In view of these experiments, we were surprised with the results obtained when solubilized A431 membranes were incubated with antisera which were not subsequently removed with Pansorbin: in these conditions, both the receptor and the heavy chains of anti-pp60^{src} IgG, but not control IgG, were phosphorylated in an EGF-stimulated manner (Fig. 2, upper panel). Antisera raised in several ASV tumour-bearing rabbits were phosphorylated by the solubilized membranes to varying extents. Those antisera which were good substrates for the solubilized EGF-stimulated kinase were also good substrates in the immunoprecipitation reaction designed to detect pp60^{src} activity (Fig. 2, lower panel). Because these antisera were selected for their ability to be phosphorylated by pp60^{src}, they did not always have parallel ability to act as substrate for pp60^{src}. Various non-immune sera failed to serve as phosphorylation substrates for either kinase. The parallel ability of the immune antisera to act as substrates for pp60^{src} and the EGF-stimulated kinase suggested that very similar determinants are required by the pp60^{src} and the EGF-stimulated kinase to allow phosphorylation of the anti-pp60^{src} sera.

The phosphoamino acids in the receptor and IgG heavy chain were analysed in phosphoproteins purified by polyacrylamide gel electrophoresis. Both the receptor and IgG were phosphorylated exclusively at tyrosine residues, and phosphate

incorporation into tyrosine in both proteins was stimulated threefold by EGF (Table 1).

The EGF stimulation of IgG phosphorylation was not due to the release of more pp60^{src} into an immunoreactive fraction because EGF failed to increase significantly the yield of pp60^{src} activity in the immune precipitate (data not shown). Furthermore, removal of a significant fraction of pp60^{src} did not alter the ability of the EGF-stimulated kinase to phosphorylate immune IgG (Fig. 3a). The inability to alter the receptor or immune IgG phosphorylation by prior immunoprecipitation of pp60^{src} suggested that these proteins were not phosphorylated by the fraction of pp60^{src} accessible to immunoprecipitation. The failure to immunoprecipitate the EGF receptor or its EGF-stimulated kinase, which is tightly associated with the EGF receptor, was not due to any impediment to binding of the Fc fragment of IgG to the protein A on the bacteria. Pansorbin could quantitatively remove antibodies which had been interacted with the EGF-stimulated kinase and phosphorylated. However, such immune precipitation did not precipitate the phosphorylated EGF receptor (Fig. 3b).

The apparent low affinity of the anti-pp60^{src} IgG for the EGF-stimulated kinase, which allowed phosphorylation but not immune precipitation, has two possible explanations. (1) The EGF-stimulated kinase could be distinct from the pp60^{src} yet similar enough to allow immune-specific recognition of antisera leading to IgG heavy chain phosphorylation. A closer homology between pp60^{src} kinase and EGF-stimulated kinase cannot be excluded. The tight association between the kinase and receptor could occlude some immune determinants, allowing the anti-pp60^{src} IgG to interact strongly enough for phosphorylation, but too weakly for immune precipitation. (2) It is possible that the phosphorylation site in the variable region of the heavy chain¹⁵ of anti-pp60^{src} contains a sequence that is preferred by tyrosine-specific kinases.

In either case, a close similarity between pp60^{src} and the EGF receptor kinase has been established. Both proteins are themselves phosphorylated at tyrosine residues and both are able to

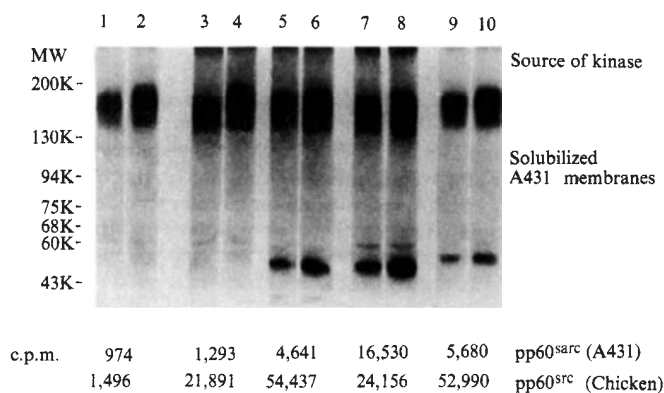


Fig. 2 EGF-stimulated phosphorylation of anti-pp60^{src} sera. Various antisera to viral pp60^{src} were raised in newborn rabbits bearing ASV-induced tumours¹⁰. Sera from newborn rabbits that did not develop tumours contained no detectable anti-pp60^{src} activity—these and several adult rabbit sera served as controls. Control sera (lanes 1, 2) and immune sera (lanes 3–10) were incubated with solubilized A431 membranes for 30 min at 0 °C as described in Fig. 1 legend, without the addition of Pansorbin. Reaction mixtures were treated with (lanes 2, 4, 6, 8, 10), or without (lanes 1, 3, 5, 7, 9), 0.3 μ M EGF followed by the addition of 15 μ M [γ -³²P]ATP for 2 min at 0 °C. Electrophoresis and autoradiography were performed as described in Fig. 1 legend. Numbers below the autoradiogram show results for the same antisera when tested for ability to precipitate, and be phosphorylated by, cellular pp60^{src} from solubilized A431 membranes and by partially purified pp60^{src} (ref. 15) from ASV-induced chicken tumours. Immunoprecipitations and phosphorylation reactions were carried out as in Fig. 1 legend except that the reactions were terminated by applying the reaction mixtures to Whatman 3M filter paper discs which were then placed in 10% TCA. The filters were washed three times in 5% TCA, then boiled in 5% TCA for 10 min; TCA and water were then removed by washing the filters in ethanol, followed by ether. ³²P incorporated into IgG heavy chains was measured by scintillation counting.

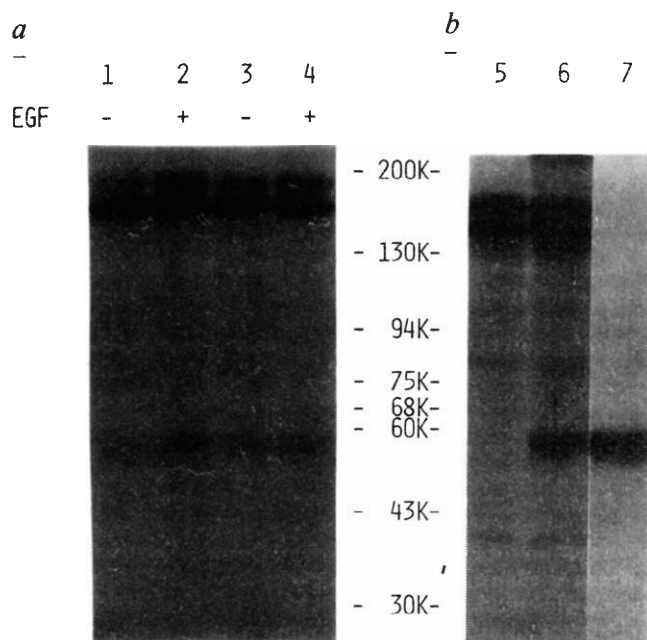


Fig. 3 a, Lack of effect of removal of pp60^{src} on EGF-stimulated phosphorylation of anti-pp60^{src} serum. pp60^{src} was removed from solubilized A431 membranes by immunoprecipitation with anti-pp60^{src} serum. The same quantity of antiserum was re-added to control (lanes 1, 2) and immune (lanes 3, 4) serum-treated supernatants. Mixtures were incubated for 30 min at 0 °C followed by addition of EGF (lanes 2, 4) or buffer (lanes 1, 3). Phosphorylation reactions, electrophoresis and autoradiography were performed as described in Fig. 1 legend. b, Failure of anti-pp60^{src} serum to precipitate the phosphorylated EGF receptor. Immune serum and EGF were added to solubilized A431 cell membranes as described in Fig. 2 legend. [γ -³²P]ATP (15 μ M) was added for 5 min at 0 °C and the reaction terminated by addition of EDTA to a final concentration of 5 mM. Immunoprecipitation was carried out using Pansorbin. Samples were analysed by electrophoresis and autoradiography. Lane 5, supernatant remaining after immunoprecipitation; lane 6, complete phosphorylation mixture; lane 7, immunoprecipitate.

phosphorylate, with parallel avidity, antisera directed against pp60^{src}, also at tyrosine residues. Although identity of the EGF-stimulated kinase with the pp60^{src} has not been established, these two kinases belong to a new family of tyrosine kinases involved in the continuum from growth control to transformation. It has been suggested that ASV transformation is an exaggerated growth response resulting from a 30- to 50-fold increase in the amount of pp60^{src} in the transformed cells¹². Although EGF stimulation does not result in transformation, sarcoma growth factor, described by DeLarco and Todaro¹⁶, does induce a transformation-like state in EGF responsive cells, apparently through binding to the EGF receptor. It is interesting to speculate that sarcoma growth factor acts as a super-agonist on the EGF receptor, increasing the tyrosine phosphorylation activity of the EGF-stimulated kinase to levels seen in ASV transformation.

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Human HLA gene segment isolated by hybridization with mouse H-2 cDNA probes

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The products of the highly polymorphic genes known as the major histocompatibility complex (MHC) have been shown to play a major part in the control of several aspects of the immune response and of susceptibility to certain diseases^{1,2}. The major transplantation antigens are membrane proteins composed of two noncovalently associated polypeptide chains: a light, non-polymorphic chain of molecular weight 12,000 (MW), called β_2 -microglobulin, and a polymorphic, glycosylated heavy chain of ~45,000 MW. The heavy chains in man and mouse are encoded in at least three loci of the MHC named HLA-A, -B and -C, and H-2D, K and L respectively. The available amino acid sequence data indicate extensive homology between human and mouse heavy chains^{3,4}. We therefore used a recently isolated cloned mouse H-2 cDNA probe⁵ to screen a human gene library. We have now characterized one of the recombinant phages obtained, λ HLA-12, and shown that it contains an authentic HLA sequence with evidence of a second one close by.

Major efforts have been directed towards the molecular cloning of HLA and H-2 gene sequences. The isolation of cloned cDNA copies has proved difficult because of the low abundance of the corresponding mRNAs *in vivo*. Nevertheless, cDNA clones carrying HLA⁶ and H-2 (ref. 5) sequences have been obtained. A recombinant plasmid, pH-2^d-3, recently isolated by Bregegere *et al.*⁷, carries a cDNA insert of 1,050 base pairs, which corresponds to the 3'-untranslated region (500 base pairs) and 550 base pairs of translated sequence of a mRNA coding for a H-2 heavy chain of the *d* haplotype. H-2 and HLA heavy chains consist of three globular domains followed by a transmembrane sequence which anchors the molecule at the cell surface, and a short intracellular carboxy-terminal fragment. pH-2^d-3 lacks the sequences coding for the first two domains but contains the rest of the coding sequence.

Comparison of HLA-B7, HLA-A2 and H-2K^b amino acid sequences shows that the third domain is the best conserved part

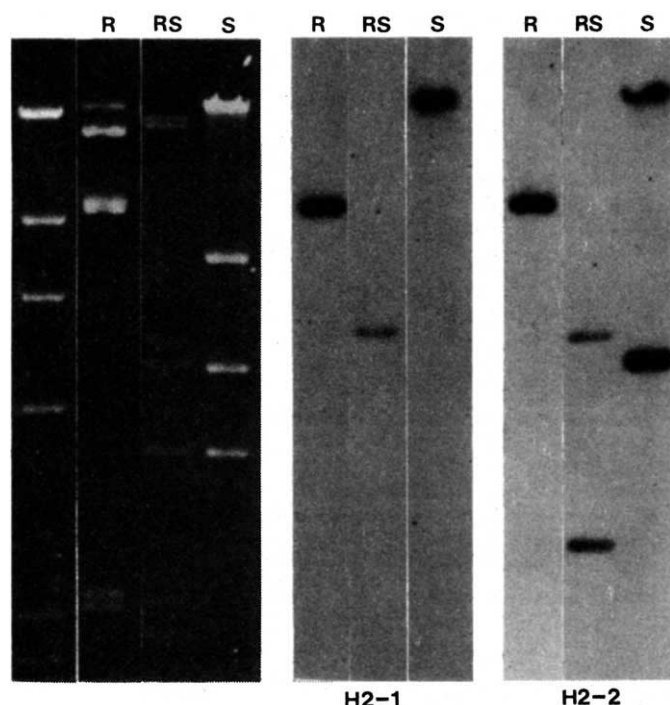


Fig. 1 Ethidium bromide-stained gel (left) and autoradiogram after transfer and hybridization with the H2-1 (middle) or the H2-2 (right) probes of λ HLA-12 DNA digested with *Eco*RI, *Sst*I and *Sst*I + *Eco*RI restriction enzymes. The first pattern on the left is *Hind*III-digested λ DNA giving fragments at 23.6, 9.46, 6.78, 4.34, 2.26 and 1.98 kilobases. Aliquots of digested λ HLA-12 DNA were run on a 0.5% agarose gel slab (overnight at 3 V cm⁻¹) and transferred to nitrocellulose according to Southern¹¹. The filter was baked at 80 °C for 2 h, pretreated for 4 h at 68 °C in 6 × SSC, 1 × Denhardt's mix¹² and hybridized overnight at 68 °C in 2 × SSC, 1 × Denhardt, 0.5 × SDS with H2-1 or H2-2 probes. The probes (*Pst*I-digested pH-2^d-3 DNA fragments) were recovered from a low gelling point agarose gel¹³, run on a small DEAE (DE52) column and labelled by nick-translation¹⁴ to specific activities of 1–4 × 10⁷ d.p.m. per μ g. After hybridization, filters were washed three times at 68 °C in 2 × SSC, 1 × Denhardt and 0.5 × SDS, twice at 20 °C in 2 × SSC, 50% formamide, 0.5% SDS, and twice in 2 × SSC. Exposure was 1–2 days at –70 °C with Ilford 'fast tungstate' screens. It is apparent from these patterns (see Fig. 2 for corresponding restriction map) that the H2-1 probe hybridizes only to the right of the last *Sst*I site in the insert whereas the H2-2 probe hybridizes to two sets of fragments which are in fact separated by ~1.9 kilobases.

in 2 × SSC (rather than the usual 68 °C). However, all positive phages hybridized strongly with probe H2-2 at 68 °C. One recombinant, λ HLA-12, containing a 15-kilobase insert, was selected for further study.

λ HLA-12 DNA was analysed by digestion with *Eco*RI, *Sst*I and *Bgl*II restriction enzymes alone or in combination. The fragments were separated on agarose gels and analysed by Southern blotting and hybridization with the H2-1 and H2-2 probes. Figure 1 shows an example of such an analysis. The restriction map obtained for λ HLA-12 is shown in Fig. 2. H2-2 hybridized to two different *Sst*I fragments, which indicates that at least one cleavage site may lie within sequences homologous to this (third domain-specific) probe; H2-1 hybridized only to the right-ward *Sst*I fragment. Hybridization to the *Bgl*II and *Bgl*II + *Sst*I fragments confirmed these results, which indicate that the gene is oriented left to right on the map.

As *Sst*I was suspected to cleave within the region coding for the third domain, λ HLA-12 DNA was digested with *Sst*I restriction enzyme and labelled at the 5' or at the 3' ends. One of the fragments hybridizing with the H2-2 probe (see Fig. 1) was isolated after secondary digestion with *Eco*RI restriction enzyme and sequenced by the Gilbert-Maxam method⁹; labelling at the 3' and 5' ends allowed sequence determination on both strands from the same restriction site.

The DNA sequence obtained to the left of the second *Sst*I site in the insert is shown in Fig. 3. The protein sequence corresponding to a translation phase open in this region is shown above the nucleotide sequence; the HLA-B7 (ref. 3) sequence is shown on top. Figure 3 shows that the region sequenced corresponds to an HLA gene segment (amino acids 199 to 230 in

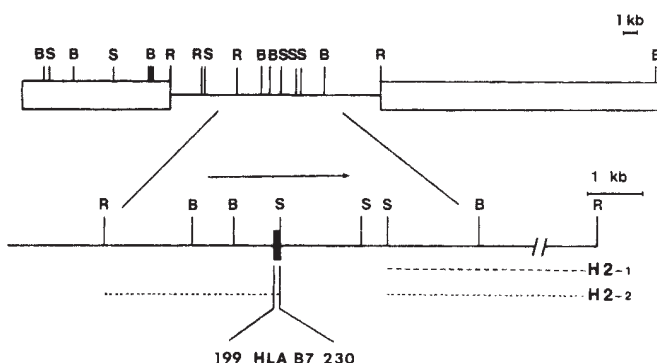


Fig. 2 Restriction map (R = *Eco*RI, S = *Sst*I, B = *Bgl*II restriction enzymes) of λ HLA-12. λ HLA-12 was identified and purified by three successive rounds of plaque hybridizations¹⁵ (1 × 10⁴, 3 × 10³ and 200 plaques per dish) using nick-translated¹⁴ (1–3 × 10⁸ c.p.m. per μ g) H2-1 or H2-2 probes (for conditions see Fig. 1 legend). Autoradiography was overnight at –70 °C using flashed films and Du Pont 'Cronex lightning plus' screens. λ HLA-12 DNA was prepared from a 600-ml culture of the *Escherichia coli* 1106 strain infected at a multiplicity of three. The phage was purified on caesium chloride step and continuous gradients and DNA was extracted using phenol, ether and ethanol precipitated. For mapping purposes aliquots (0.5 μ g) of the DNA were digested with restriction enzyme(s) and analysed in parallel on 0.5% agarose gel slabs. Southern hybridizations¹¹ were done as described in Fig. 1 legend. Top, complete map including the Charon 4A λ phage¹⁶ (with the right arm to the left to show the insert in the correct orientation with respect to gene sequences). Bottom, enlargement of the region in which DNA sequencing was performed, the direction of transcription is shown by the arrow. The dashed line indicates the *Eco*RI–*Sst*I fragment hybridizing with H2-1, the dotted line those hybridizing with H2-2. The area whose sequence is given in Fig. 3 is indicated by the box; the corresponding region in the HLA-B7 protein sequence is shown below with the numbers of the terminal amino acids. The approximate position for the third *Sst*I site in the insert is indicated.

of the molecule^{3,4}; the H-2^d amino acid sequence derived from the pH-2^d-3 DNA sequence shows 72.6% homology with that of HLA-B7 in the third domain⁷. These observations led us to speculate that there might be sufficient nucleotide sequence homology between H-2 and HLA DNA sequences to allow specific hybridization of H-2 probes to human HLA genomic segments.

Two DNA fragments were purified from the pH-2^d-3 plasmid after digestion with *Pst*I: one (abbreviated H2-1) is ~600 base pairs long and includes sequences coding for the transmembrane and cytoplasmic parts of the H-2 heavy chain and the other, H2-2, is 195 base pairs long and encodes most of the third domain⁷. Both fragments were labelled with ³²P by nick-translation and used as probes to screen a human gene library consisting of random DNA fragments recombined in λ Charon 4A, constructed by Lawn *et al.*⁸. About 10⁵ plaques were screened and several positive phages were purified. *In situ* hybridization was initially carried out with probe H2-1 at 63 °C

Fig. 3 Sequence of 96 nucleotides located immediately to the left of the second *Sst*I site (see Fig. 2). Bottom line, sequence of the DNA strand corresponding to the mRNA (determined independently on both strands using kinase labelling for the 5' end and terminal transferase-cordycepin labelling¹⁷ for the 3' end); middle line, deduced protein sequence; top line, HLA-B7 sequence¹⁰

HLA B*7:01:199
A T L R C W A L G F Y P A E I T
A T L R C W A L G F Y P A E I T
5' G C C A C C C T G A G G T G C T G G G C C C T G G G C T T C T A C C C T G C G G A G A T C A C A

HLA B*7:01:230
L T W Q R D G E D Q T Q D T E L
L T W Q R D G E D Q T Q D T E L
C T G A C C T G G C A G C G G G A T G G G G A G G A C C A G A C C C A G G A C A C G G A G C T (C) 3'
↑
SstI

the third domain region) because the two protein sequences coincide exactly.

Matching the H2-2 probe sequence⁷ to the HLA sequence in Fig. 3 reveals 89% homology over the 96 nucleotides shown. This degree of homology, if valid over longer distances, is compatible with the cross-hybridization observed between H-2 and HLA sequences in the rather stringent conditions used.

H-2 cDNA probes have thus allowed us to identify at least one genomic clone containing authentic HLA sequences. The sequence determined shows complete identity with the published HLA-B7 data^{3,10}. However, as HLA-B7 and HLA-A2 are identical in this area we do not know the locus from which the cloned segment originates; further sequencing may clarify this point. The human library from which these clones were obtained was constructed using DNA from a human fetus of unknown HLA phenotype⁸; it will be useful to screen libraries constructed with DNA from individuals of known haplotype with the HLA probes obtained in this study.

Whether or not λ HLA-12 carries a complete HLA gene is also an open question; the restriction map (Fig. 2) shows that the cloned segment includes nearly 8 kilobases of DNA upstream from the third domain coding region, that is, potentially a complete HLA gene.

The hybridization pattern observed in the Southern blot (Fig. 1) and the map location of the corresponding fragments (Fig. 2) strongly suggest that the λ HLA-12 insert actually contains two sets of HLA-coding sequences. This would indicate tandemly arranged HLA genes or gene segments and would have important implications for the organization of the histocompatibility gene cluster.

Further analysis of λ HLA-12 and other clones isolated from the same gene library should elucidate the structure of HLA genes. It will also provide the tools and probes to investigate the structure of genes of defined haplotypes and the genetic origin of the polymorphism observed for HLA products.

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Direct role of the *himA* gene product in phage λ integration

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The integration of phage λ into the *Escherichia coli* chromosome is accomplished by a site-specific recombination between two unique DNA sequences¹ (*attB* on the bacterial genome and *attP* on the phage; reviewed in refs 2, 3) and requires proteins encoded by both the bacterium^{4,5} and the phage⁶⁻⁹. Genetic and biochemical studies have shown that bacterial strains mutant in the *himA* gene, located at 38 min on the *E. coli* map, are defective in the activity of the host-encoded component¹⁰⁻¹². They are, moreover, defective for the growth of bacteriophage Mu, for precise excision of transposable antibiotic resistance determinants¹³ and for the synthesis of the λ *int* gene product¹⁴. We now show that the *himA* gene product (*phimA*) is not solely a regulator of genes involved in integration but is one of two host polypeptides required for integrative recombination.

To identify the *himA* gene product, we used specialized λ transducing phage carrying the *E. coli himA* gene and derivatives of this phage carrying missense or nonsense mutations¹³. We infected heavily UV-ray irradiated bacteria with λ *himA* transducing phage and labelled the proteins made with radioactive amino acids¹⁵. The UV rays destroy the capacity of the bacteria to direct the synthesis of host proteins and thus only proteins encoded by the transducing phage are synthesized. The bacteria are lysogenic for a derivative of λ that is not inducible by UV rays. This prevents transcription from the phage promoters. Therefore, proteins encoded by the bacterial DNA sequences carried by the phage are selectively synthesized.

Figures 1 and 2 show the results of such experiments performed with λ himA⁺, λ himAam79 (an amber mutation in the *himA* gene) or λ himA42 (a missense mutation). Labelled

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extracts were subjected to two-dimensional gel electrophoresis. Figure 1a, b shows autoradiograms of gels of bacteria infected with λ himA⁺ and λ himAam79, respectively. The two autoradiograms are equivalent except for the absence, in the gel of the λ himAam79-infected cells, of an 11,000-molecular weight (MW) spot that is present in the gel of bacteria infected with λ himA⁺. Gels of extracts of uninfected cells were devoid of labelled spots (data not shown). As nonsense mutations are known to cause premature polypeptide chain termination, the absence of this spot tentatively identifies this 11,000-MW protein (marked himA in Fig. 1a) as the product of the himA gene (phimA). No protein that could correspond to the phimAam79 amber fragment is seen. Note that the phimA spot seems to exhibit charge heterogeneity in the isoelectric focusing dimension. The separation of two apparent species of phimA varies somewhat with the conditions of electrophoresis.

Further evidence supporting the identification of the 11,000-MW protein as phimA comes from an additional experiment detailed in Fig. 2. In this experiment, the dose of UV rays was insufficient to eliminate completely all host protein labelling and a small amount of label was incorporated into ribosomal protein S10 which therefore serves as a convenient reference point (see

below for the location of S10 in this gel system). As in the previous experiment, cells infected with λ himAam79 (Fig. 2a) lack the 11,000-MW phimA protein that is made after infection with λ himA⁺ (Fig. 2b). Furthermore, the λ himA42 transducing phage directs the synthesis of a protein of identical molecular weight to phimA but with a more basic charge. This suggests that the himA42 mutation has substituted an amino acid in the phimA protein that results in a net basic charge shift.

From the results of the experiments discussed above, we identify phimA as an 11,000-MW protein. The position of this protein in the isoelectric focusing indicates that phimA is a weakly basic protein²⁸. We do not know whether the charge heterogeneity of phimA reflects the state of this protein within the cell.

The λ himA transducing phage directs the synthesis of several proteins in addition to phimA, most of which have previously been identified by Springer *et al.*¹⁷ and Henneke *et al.*^{18,19}. These workers used the same transducing phage in similar experiments, but analysed the labelled proteins by one-dimensional polyacrylamide gel electrophoresis. We were thus able to identify the spots in two-dimensional autoradiograms by comparing their apparent molecular weights and relative intensities. In Fig.

Fig. 1 Autoradiograms of two-dimensional gels of UV ray-irradiated lysogenic bacteria infected with λ himA⁺ (a) or λ himAam79 (b). Logarithmic phase cultures of strain K447 (159uvrA⁺ carrying λ c⁺ind⁺Nam7, 53) growing at 39 °C in M56 media²⁶ plus 0.2% maltose at a concentration of 2×10^8 ml⁻¹ were pelleted and resuspended at a concentration of 2×10^9 ml⁻¹ in M56 media. Cells (10 ml) were irradiated with 1.6×10^4 ergs mm⁻² of UV light from a GE germicidal lamp (~2 min at a distance of 10 cm), pelleted, resuspended to the same concentration in M56 with 0.02 M MgSO₄ and incubated in the dark for 15 min with shaking at 37 °C. To 0.5 ml of these cells, phage were added at a MOI of 10, and adsorption was allowed on ice for 15 min. The infected cells were once again pelleted and the supernatant discarded. To initiate growth, 2.5 ml of pre-warmed M56, 0.2% maltose and 0.02 M MgSO₄ were added and the cultures shaken at 37 °C in the dark. After 5 min, 10 μ Ci of ¹⁴C mixed amino acids (55 mCi mmol⁻¹, NEN) were added and labelling was allowed to continue for 60 min. The cells were then washed with cold 0.01 M Tris pH 7.4 and 0.01 M MgCl₂ and prepared for isoelectric focusing (IF). Strain K37 and the λ himA transducing phage are described by Miller and Friedman¹². Phage stocks were prepared by lytic growth in K37. Lysates were concentrated by polyethylene glycol precipitation²⁷ and purified by isopycnic CsCl buoyant density centrifugation. The phage were dialysed against 0.01 M Tris pH 7.4 and 0.01 M MgSO₄. Two-dimensional non-equilibrium gels were run according to the method of O'Farrell²⁸. All recipes and reagents are described in ref. 16. The IF dimension was run for 1,400 V-h using LKB ampholines (pH 3.5–10). The second dimension was a 20% polyacrylamide SDS stacking gel system with a 23-mm stacking gel and a 90-mm separating gel. Second dimensions were run for 5 h at 3.3 W per gel. The gels were stained in 0.005% Coomassie brilliant blue in 0.01% trichloroacetic acid, 7.5% acetic acid and destained in 7.5% acetic acid. Most of the ampholines could be removed by first soaking the gel in stain made 50% with ethanol. The gels were hot vacuum dried on Biorad paper and arbitrary orientation marks were drawn with radioactive india ink to facilitate subsequent alignment of the gel with the autoradiograms. The gels were then exposed to Kodak X-Omat film. Spot identifications are described in the text. The arrow in b indicates the expected location of phimA. Exposure time was 60 d. Samples were prepared for IF in the following manner. The labelled cultures were pelleted in 1.5-ml Eppendorf tubes; 100 μ l of 0.01 M Tris pH 7.4 and 0.01 M MgCl₂ containing 100 μ g ml⁻¹ freshly prepared lysozyme (Worthington) were added and the pellet resuspended. The cells were freeze-thawed for five cycles in an ethanol-dry ice bath.

Aliquots of 5 μ l of a 1 mg ml⁻¹ solution each of pancreatic DNase (Worthington) and RNase (Sigma) were added and incubated on ice for 10 min. Then, 50 mg of solid urea (Schwarz-Mann ultrapure) and 100 μ l of lysis buffer¹⁶ were added to complete the sample preparation. The extracts were stored at -80 °C. The molecular weights of proteins were estimated by comparing the mobilities of the proteins in the second (SDS polyacrylamide) dimension with the mobilities of known proteins: two internal standards whose MWs are known from primary structure determinations were IF3 (20,668)²⁹ and whose location was determined in this work; and *E. coli* HU protein (9,535)³⁰, identified with purified HU protein (from K. Abremski). An additional marker was ribosomal protein S10 taken as 10,500 (refs 31, 32).

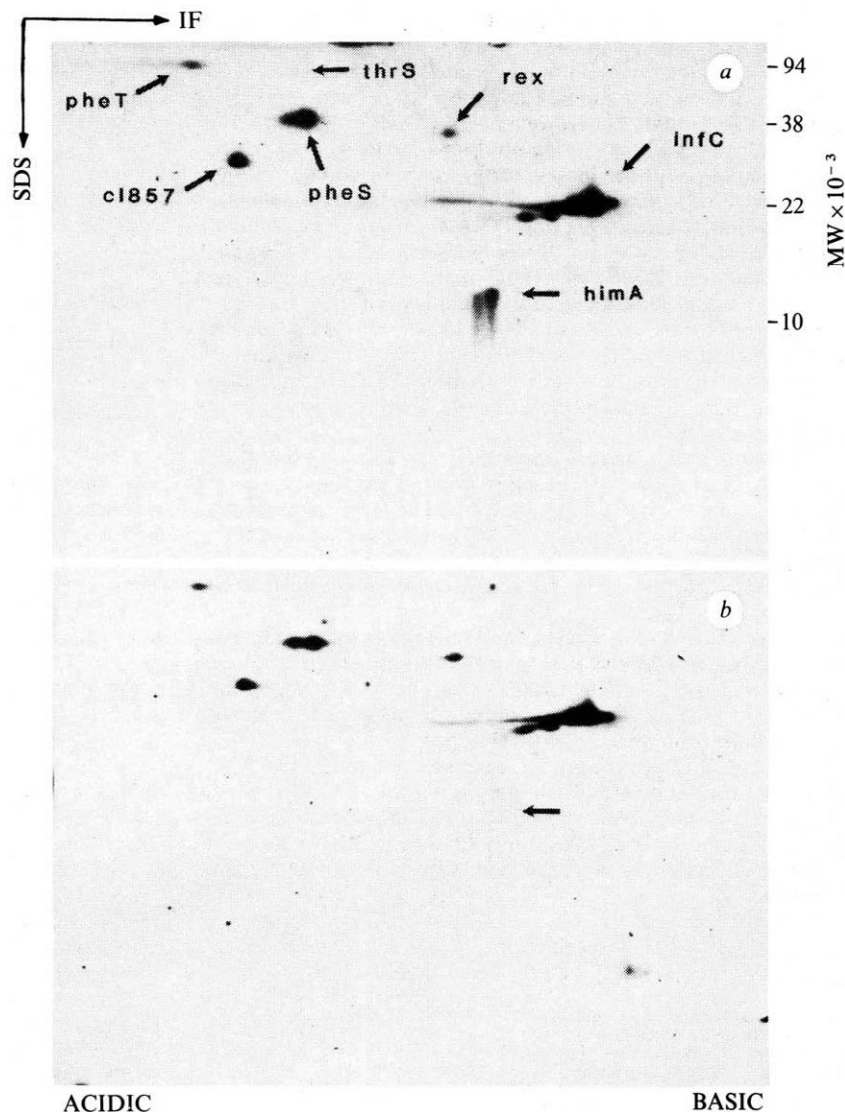
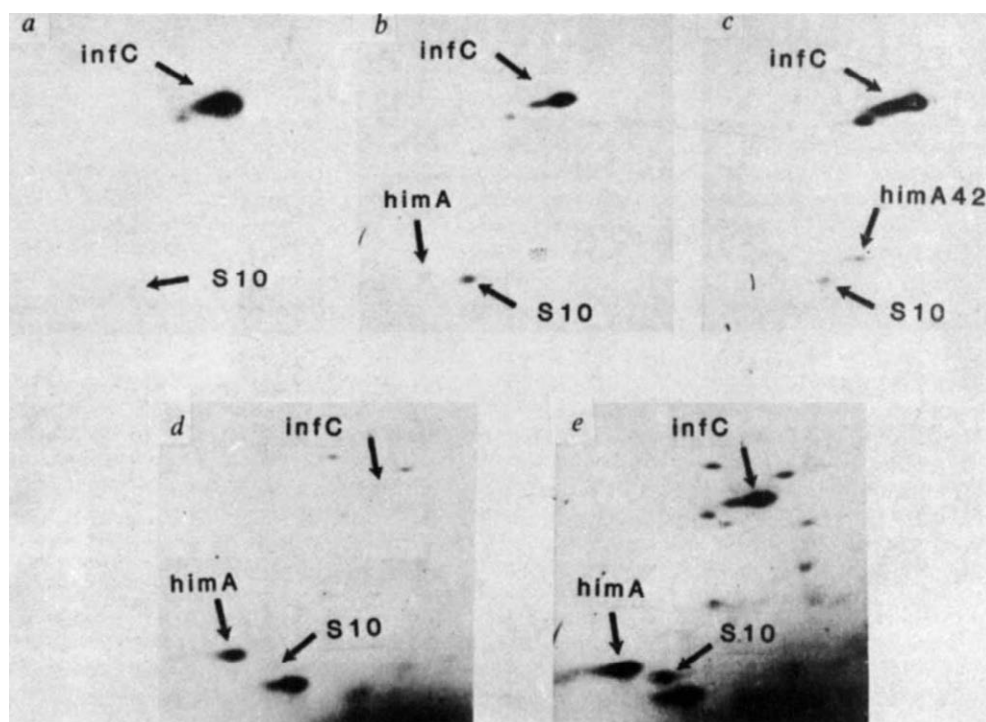


Fig. 2 Details of gels and autoradiograms of the regions surrounding S10 and IF3. *a-c*, UV-ray irradiated bacteria infected with λ himAam79 (*a*), λ himA⁺ (*b*) and λ himA42 (*c*). All three are autoradiograms. *d* Is the stained gel used to make the autoradiogram in *b* with the addition of purified IHF. *e* Is a composite overlay of *b* on *d*. Exposure time was 60 d. Purified integration host factor (IHF) was concentrated by precipitation with 20% trichloroacetic acid and resuspended in a 1:1 mixture of lysis buffer and 8 M urea.



1a, the spots are named for their respective genes. Thus, the spot marked *infC* is translation initiation factor IF3. As noted by Springer²⁰, the *infC* spot is the most heavily labelled and consists of a cluster of spots due to the different species of IF3²¹. We were able to locate the spot for IF3 in two-dimensional gels of total *E. coli* proteins by overlaying this autoradiogram on the stained gel. IF3 maps at coordinates (42, 54) on the standard non-equilibrium two-dimensional gel map of *E. coli* proteins of Bloch *et al.*²². The spots marked *thrS* (threonyl-tRNA synthetase), and *pheS* and *pheT* (the α - and β -subunits of phenylalanyl-tRNA synthetase) are also shown (the *pheT* spot does not resolve well in this system). Two additional spots have been identified as the products of the λ *cI857* (repressor) and *rex* genes. These proteins, which are the only λ proteins synthesized in a repressed lysogen, were identified in separate experiments using non-transducing λ phage (H.I.M., in preparation).

Springer *et al.*¹⁷ have reported the presence of a fifth protein, p12, that they could not associate with a known gene. We have also found this protein in one-dimensional gels of our extracts. It is not *phimA* as it does not disappear when λ himAam79 is used for infection. (We were unable to detect *phimA* in the one-dimensional system, presumably due to the high background found in the region where *phimA* should migrate.) Furthermore, the p12 band does not resolve into a distinct protein spot in two-dimensional gels, indicating that it may not constitute a unique protein species as suggested by Hennecke *et al.*¹⁸.

The *E. coli* component required for integrative recombination has been extensively purified (H. A. N. and C. Robertson, in preparation). This protein (IHF) was assayed by its ability to complement purified Int protein²³ for *in vitro* recombination. The most highly purified material contains two polypeptides, and available evidence indicates that both are required for recombination activity. In the experiment shown in Fig. 3, 5 μ g of purified protein (fraction V) was mixed with a small amount of ¹⁴C-labelled total *E. coli* proteins (as reference proteins). Figure 3a shows the stained gel, *b* the autoradiogram and *c* a composite overlay of the autoradiogram and the stained gel. As shown in Fig. 3a, purified integration host factor (IHF) consists of two polypeptide species of molecular weights 11,000 (α , marked *himA*) and 9,500 (β , arrow). Both species exhibit apparent

charge heterogeneities in this gel system. The intensity of staining indicates an approximately equal molar amount of α and β . Note that the position of the 11,000-MW spot roughly corresponds to the position of *phimA* in Fig. 1a. In addition, both *phimA* and α exhibit similar patterns of heterogeneity.

Figure 3b shows the location of total *E. coli* protein labelled with ¹⁴C mixed amino acids. We have indicated the location of two reference proteins, IF3 (marked *infC*, see above) and ribosomal protein S10²². Note in Fig. 3b that although neither subunit is present in detectable quantities, S10 is positioned between the α - and β -subunits of IHF. Figure 2b, d, e shows the results of an experiment in which purified IHF was mixed with radiolabelled *phimA* (from a UV-ray irradiation experiment) and the mixture was subjected to gel electrophoresis. Only details of gels including the regions surrounding IF3 and S10 are shown. Figure 2d shows the stained gel, *b* the autoradiogram and *e* the composite overlay. The additional spots seen in Fig. 2d and *e* are unlabelled *E. coli* proteins derived from the extracts of the UV-ray irradiated bacteria. The radiolabelled *phimA* protein co-migrates with the purified α -subunits of IHF.

Thus, the *himA* genes codes for one of the subunits of IHF active in integrative recombination by λ DNA *in vitro*. This result fully explains the fact that strains mutant in the *himA* gene lack an active integration host factor and also eliminates the possibility that the *himA* gene influences λ integration solely at the level of synthesis of components of the recombinational apparatus. The demonstration that *phimA* is directly involved in integrative recombination has gained considerable importance in light of the recent finding that the *himA* gene also regulates λ integration at the level of synthesis of Int protein¹⁴. A feature of the transposon Tn3-encoded *tnpR* gene product is its involvement both in recombination and in control of transcription of transposon genes^{24,25}. Thus, dual function of site-specific recombination proteins may be a common characteristic.

The *E. coli* gene coding for the β -subunit of IHF has not been determined, although several candidates are available⁹ (H.I.M., in preparation). However, one or more of these gene products may be involved indirectly by regulating the synthesis or processing of either of the active subunits of IHF.

The fact that *phimA* is directly involved in recombination raises the possibility that *phimA* might also directly participate

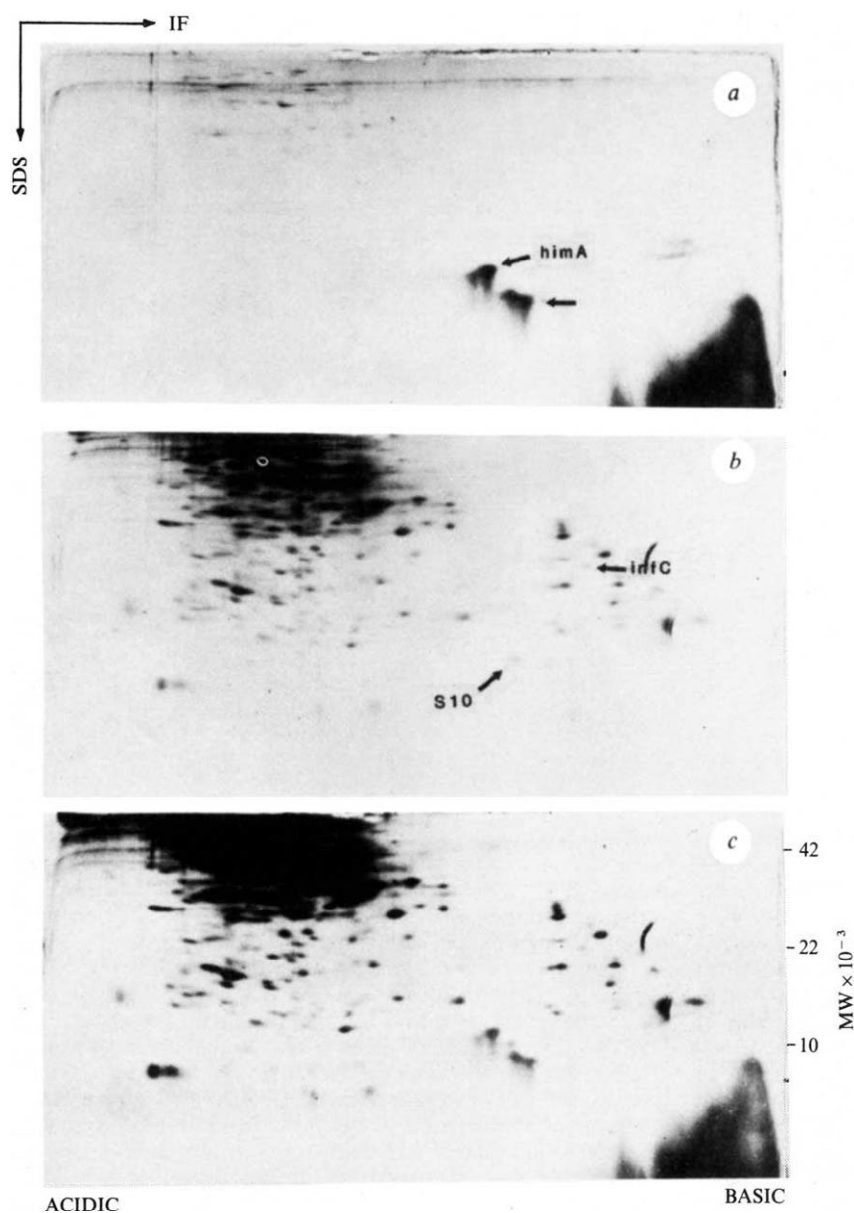


Fig. 3 Two-dimensional gel of purified integration host component. *a*, Coomassie blue stained gel. *b*, Autoradiogram of ^{14}C -labelled total *E. coli* proteins added as internal markers. *c*, Composite overlay of autoradiogram and stained gel. Additional spots in *a* are due to added *E. coli* proteins. Exposure was for 10 days. Dark staining material in bottom right of *a* is residual ampholines. For labelling bacterial cultures, $10\ \mu\text{Ci}$ of ^{14}C mixed amino acids were added to a logarithmic phase culture at a cell density of $2 \times 10^8\ \text{ml}^{-1}$ in M56 media and 0.2% glucose. After 2 min at 37°C , $100\ \mu\text{l}$ of 30% cassamino acid solution was added to the 2-ml culture and the cells were prepared for isoelectric focusing.

in several diverse processes known to be influenced by *himA* mutations, such as the growth of bacteriophage Mu and the precise excision of transposable antibiotic resistance elements. The availability of purified IHF should facilitate the biochemical investigation of these processes.

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A non-disruptive technique for loading calcium buffers and indicators into cells

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Present methods for measuring or buffering intracellular free calcium concentrations are almost entirely limited to robust and well anchored cells which can tolerate insertion of ion-selective microelectrodes or microinjection of calcium indicators or buffers into one cell at a time¹. A very few types of small cells can be loaded with buffers or indicators during controlled lysis²⁻⁴, but such procedures grossly perturb membrane integrity and soluble cytoplasmic constituents. Liposome fusion releases only trace quantities of the trapped solute into the cytoplasm^{5,6} and incorporates foreign lipid into the target cell membranes. I now describe a simple technique which loads Ca^{2+} -selective chelators into the cytoplasm of intact cells in suspension and avoids the disadvantages of previous methods. The chelators are made temporarily membrane permeable by masking their four carboxylates with special esterifying groups which then hydrolyse inside the cells, regenerating and trapping the original chelators. The method is demonstrated on red cells, mast cells and lymphocytes.

The Ca^{2+} -specific chelator used in initial trials (shown in Fig. 1a) is a member of a homologous family^{7,8} of buffers and optical indicators that have unusual specificity for Ca^{2+} over Mg^{2+} , H^+ , Na^+ and K^+ . Its affinity constant for Ca^{2+} is $\sim 10^7 \text{ mol}^{-1}$; other members of the family offer both higher and lower affinities. The simplest esters (methyl or ethyl) of this chelator are not hydrolysed in human erythrocytes. Anhydrides, imidazolides, or activated aryl esters were rejected because the end products would probably include chelator molecules irreversibly bound to intracellular nucleophiles. Trimethylsilyl esters were too rapidly hydrolysed extracellularly, while *t*-butyldimethylsilyl esters were potentially haemolytic. Success was finally achieved with the acetoxymethyl tetraester (Fig. 1b); analogous acyloxyalkyl esters are used to enhance intestinal absorption of antibiotics^{9,10}, and the accepted mechanism of hydrolysis is enzymatic cleavage of the acetyl ester linkage, followed by rapid spontaneous release of formaldehyde, followed by rapid spontaneous release of formaldehyde. Acylesterases are present in many cell types, as suggested by their ability to hydrolyse fluorescein diacetate^{11,12}.

The acetoxymethyl ester (Fig. 1b) was prepared by dissolving the chelator free acid, 5 equivalents of dry ethyldiisopropylamine (redistilled from reagent supplied by Aldrich Chemicals) and 6 equivalents of acetoxymethyl bromide¹³ in ethanol-free chloroform. After stirring for 24 h at room temperature, the solution was evaporated under vacuum and the residue dissolved in benzene, filtered to remove amine salts, and re-evaporated. Extraction of this residue with ether, followed by evaporation, yielded a soft gum which was largely but not entirely pure as determined by PMR spectroscopy. Solutions or

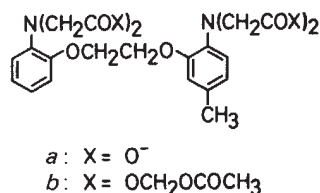


Fig. 1 Structures of the calcium chelator (a) and its acetoxymethyl tetraester (b). Hydrolysis of the ester should yield the chelator tetra-anion, four molecules each of acetate and formaldehyde, and eight protons.

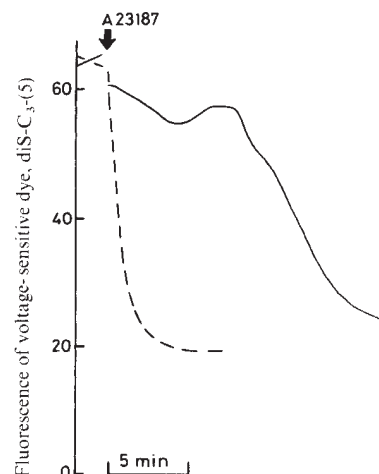


Fig. 2 Ability of intracellular chelator to delay the onset of the Ca^{2+} -induced K^+ permeability caused by A23187 (added as indicated to a final concentration of 200 nM). Abscissa, time; ordinate, fluorescence from 250 nM of the voltage-sensitive dye diS-C₃(5)^{18,19} in a suspension (0.04% haematocrit) of either normal human red cells (broken line) or cells loaded with chelator as described in the text (solid line). Fluorescence (excitation $630 \pm 10 \text{ nm}$, emission $665 \pm 10 \text{ nm}$) is expressed as a percentage of that seen in the absence of cells. The medium contained 130 mM NaCl, 10 mM KCl, 0.1 mM CaCl_2 and 10 mM tris(hydroxymethyl)-aminomethane-morpholinopropanesulphonate, pH 7.4, 22 °C. Decreased dye fluorescence indicated membrane hyperpolarization due to increased K^+ permeability, resulting from increased $[\text{Ca}^{2+}]_i$. Ester treatment was followed by sufficient washing to remove essentially all acetate and formaldehyde. The low concentration of A23187 was adequate to induce K^+ permeability because of the absence of Mg^{2+} from the medium¹⁵. At an external $[\text{K}^+]$ of 10 mM, the effect of the dye on K^+ permeability may be disregarded¹⁷.

suspensions of the ester in physiological saline were prepared by injecting freshly made-up concentrated stock solutions in dimethyl sulphoxide into well stirred aqueous solutions.

Human erythrocytes were used in initial trials as they are readily available in large numbers, lack internal Ca^{2+} -sequestering organelles and proteins, and have a well characterized Ca^{2+} -triggered K^+ permeability. The low rate of endogenous H^+ production and the rapidity of transmembrane pH equilibration allow ester hydrolysis to be monitored by H^+ production in a pH-stat. There was no ester hydrolysis detected at neutral pH in the absence of cells, but with cells at 10% haematocrit, pH 7.4, 37 °C, the partially purified ester (0.2 mg per ml of suspension) released protons at a rate decaying roughly exponentially with a time constant of $\sim 15 \text{ min}$. After 2 h, total acid production, corrected for endogenous H^+ production, was 1.56 mM, slightly more than expected from complete hydrolysis of perfectly pure ester. Cell lysis after 2 h was 5.5%, partly due to the continued vigorous stirring necessary in a pH-stat. Titration of an aliquot of the cell suspension with CaCl_2 and a Ca^{2+} -selective electrode¹⁴ showed that the amount of chelator accessible to the extracellular space was only $\sim 3\%$ of that expected from total ester hydrolysis. About this amount would be expected just from haemolysis during the loading procedure. However, once the cells were lysed by freezing and thawing, titration revealed $\sim 1.4 \text{ mmol}$ chelator per litre of cells ($\sim 90\%$ yield) with the correct Ca^{2+} affinity, within experimental uncertainty imposed by incomplete Ca^{2+} selectivity of the electrode at $p\text{Ca} \geq 7$. The amount of intracellular chelator did not significantly change either after resuspension of the loaded cells in chelator-free saline for 1 day, or after digestion with 0.1 M KOH for 2 days, showing that the chelator was well trapped and that hydrolysis was complete. Imperfect purity of the ester gum was probably a major reason why the yields of protons and chelator were not quite stoichiometric.

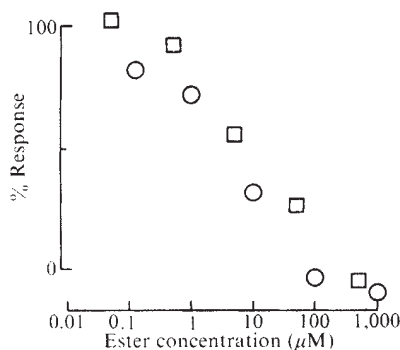


Fig. 3 Dose-response curve showing the ability of intracellular chelator to counteract the stimulation of histamine secretion from rat peritoneal mast cells by Con A plus lysooleoylphosphatidylserine. The cells were obtained and purified as described elsewhere²¹. The lysolipid was a gift of Dr G. A. Smith. The abscissa indicates the concentration of ester in which each batch of cells (10^5 per $25 \mu\text{l}$) was incubated for 30 min at 25°C . The cell suspensions were then diluted to $500 \mu\text{l}$ each, stimulated with Con A and lysolipid (final concentrations $100 \mu\text{g ml}^{-1}$ and $1 \mu\text{M}$ respectively) for 15 min, and assayed for histamine release²¹. The ordinate indicates histamine release (corrected for spontaneous release) as a percentage of the stimulated histamine release in the absence of ester. This percentage is negative for ester concentrations $\geq 100 \mu\text{M}$ because cells thus treated released less histamine after stimulation than the 'spontaneous release' controls that were neither stimulated nor treated with ester. Circles and squares represent two independent experiments in which spontaneous histamine release was, respectively, 2.9 and 5.0% of total cell histamine, and stimulation without ester gave releases of 23.6 and 11.0%. Control experiments showed that the inhibition by ester was not due to formaldehyde or acetate, as preincubation with up to $400 \mu\text{M}$ formaldehyde (as would be produced by complete hydrolysis of $100 \mu\text{M}$ ester) or 4 mM acetate showed either no effect on, or an actual enhancement of, stimulated histamine secretion.

Figure 2 shows an independent demonstration that these cells have trapped a cytoplasmic high-affinity Ca^{2+} chelator. In normal cells (broken line), addition of the Ca^{2+} ionophore A23187 causes an influx of Ca^{2+} , increasing the intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$) and switching on a K^+ permeability^{2,15,16}. In medium containing 10 mM K^+ (ref. 17), this permeability hyperpolarizes the cells and can be detected as the decrease in fluorescence (ordinate) of a membrane potential-sensitive dye^{18,19}. Introduction of an intracellular Ca^{2+} chelator should mean that the Ca^{2+} influx has to continue much longer before the threshold $[\text{Ca}^{2+}]_i$ is reached. As expected, the ester-treated cells (solid line) show a marked delay before hyperpolarization develops, presumably reflecting the time required for the Ca^{2+} influx to titrate the trapped buffer.

Another cell type that accumulates chelator is the rat peritoneal mast cell, but the small number of cells and their internal complexity made use of the pH -stat and direct Ca^{2+} titrations impractical. Instead, chelator uptake was qualitatively assessed by the inhibition of $[\text{Ca}^{2+}]_i$ -dependent histamine release²⁰. Figure 3 shows that preincubation of mast cells with increasing concentrations of the ester progressively inhibited histamine release in response to concanavalin A (Con A) plus lyso-phosphatidylserine^{21,22}. Preincubation with ester was also able to stave off the normally immediate release of histamine induced by a moderate dose of A23187. However, higher concentrations of the ionophore or longer exposure times eventually overcame this inhibition and led to the release of most of the cellular histamine. Thus the effect of preincubation with ester is consistent neither with a blockage of Con A-induced Ca^{2+} permeability, nor with permanent fixation of the histamine granules or metabolic poisoning, but can be explained by a fixed quantity of high-affinity chelator preventing or delaying the rise of $[\text{Ca}^{2+}]_i$ to the threshold for secretion. No toxic effects have yet been

detected in red blood cells or mast cells from the formaldehyde which should be released by the hydrolysis of effective concentrations (up to several hundred μM) of ester. This is probably because the high permeability of formaldehyde and the low cytotoxicity prevent its concentration exceeding millimolar amounts anywhere. Histological fixative concentrations are of the order of hundreds of millimolar, whereas for short-term exposures to several millimolar, mild and partially reversible pharmacological effects are reported^{23,24}. If formaldehyde toxicity presents difficulties, it should be possible to change the acyloxy-alkyl group to release alternative carbonyl compounds^{9,10}.

These experiments show how chelators trapped by ester hydrolysis can damp out short-term $[\text{Ca}^{2+}]_i$ transients and thus reveal qualitatively which cell functions are influenced by such transients. The method described here can be greatly extended by choosing a chelator which is also a sensitive optical indicator of $[\text{Ca}^{2+}]_i$. For example, a fluorescent methoxyquinoline (structure 3b of ref. 7) is readily trapped inside mouse thymocytes and pig mixed lymphocytes by its acetoxymethyl ester²⁵. At low indicator dose, the Ca^{2+} sensitivity of its fluorescence⁷ shows that resting $[\text{Ca}^{2+}]_i$ in lymphocytes is $123 \pm 6 \text{ nM}$ ($\pm \text{s.e.m.}$, $n = 27$), a much sought-after²⁶ but hitherto unmeasured value. Higher intracellular concentrations of chelator, up to at least 4.7 mM , are also easily attained by adjustment of cytochrome and initial ester concentration. Effects of such buffering dosages on capping and mitogenic stimulation are being studied; the fluorescence sensitivity allows quantification of the effectiveness of the buffer in damping changes in $[\text{Ca}^{2+}]_i$.

Intracellularly hydrolysable esters offer the first method for trapping membrane-impermeable chelators and indicators inside large populations of small intact cells without disrupting their membranes. A technically simple and reliable new approach to the measurement and control of $[\text{Ca}^{2+}]_i$ thus becomes possible. All cell types tested so far have proved able to accumulate significant amounts of chelator. As this technique has been shown to give the expected results in cell types where the role of intracellular Ca^{2+} is fairly well understood, it can now be applied with greater confidence to a wide variety of tissues where the function of Ca^{2+} is less well established. Analogous esters may also prove useful outside the field of Ca^{2+} function, for example to deliver and trap indicators of pH and free Mg^{2+} , chelators of toxic heavy metals, or other membrane-impermeable carboxylic acids.

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BOOK REVIEWS

Memories of the secret city

Rudolf Peierls

LOS ALAMOS was the laboratory set up, during the Second World War, to design and produce atomic bombs from the fissile materials, uranium 235 and plutonium, provided by the gigantic installations of the "Manhattan District" of the US Army. A remote location in the hills of New Mexico was chosen partly because of the high degree of secrecy required, and partly because the work might involve hazardous experiments. Conceived originally as a settlement for 265 people — six scientists with their support staff and families — it had grown to many thousands by the end of the War, largely because the design of a plutonium bomb proved much more difficult than had been foreseen.

Much has been written about the achievement of the laboratory in solving the difficult technical problems so quickly, and about the life in this unusual community. The present collection of lectures, given at Santa Barbara in 1975, is a most interesting addition to this literature, containing the recollections of scientists, of scientists' wives and of an army officer.

Imagine a team of scientists, many of them at the top of their professions, working in improvised buildings, living in temporary housing, depending for their professional and domestic needs on the army, and subject to security restrictions they did not always see as necessary or consistent; imagine a collection of army personnel supporting the activities of scientists whose objectives they were not supposed to know, and whose methods and manners were quite foreign to the army's style of operation. There were bound to be conflicts, irritations and grievances. The recollections indeed include many examples of such friction. The fact that all major problems were somehow resolved, and the minor nuisances accepted, is a tribute to the good sense of the people involved, their feeling of urgency and in no small measure to the personality of the director, Robert Oppenheimer.

There is already some folklore about the place. The first lecture, by General Dudley, reports four versions of how Los Alamos came to be selected as the site of the laboratory — his own and three others — of which at least two are quite incompatible with his and with each other. The recollections of the residents do not show any such disagreements on facts, but they differ much in emphasis. Some seem to remember above all the irritations, the shortages, the beautiful school building which for a time had no qualified teachers, the strange facets of the voluntary mail

Reminiscences of Los Alamos, 1943–1945. Edited by Lawrence Badash, Joseph O. Hirschfelder and Herbert P. Broida. Pp.188. (Reidel: 1980.) Hbk Dfl.50; pbk Dfl.20.

censorship. Others stress the attractions of the landscape and climate, the contacts with the local Indian culture, and above all the proximity of a large number of unusually gifted and interesting people. But all would probably agree with Laura Fermi's remark:

One interesting thing to me is that during the war I was in Los Alamos only a year and a half, and *still* it seems such a big portion of my life. . . . It was so different, it was such intense living, that it seems impossible that in my case it actually lasted hardly eighteen months.

Her account and those of Bernice Brode and Elsie MacMillan give a lively picture of the domestic scene. Feynman's "Los Alamos from Below" is as entertaining as one has come to expect from him. He reveals the secret of his notorious skill in opening combination locks, and how he accidentally stumbled on a safety hazard in a complicated plant design. There are hilarious accounts of his brushes with the censor and other bureaucratic elements. In stressing his "low" status he has his tongue in his cheek. He was recognized at once as a man of outstanding promise. The opening statement that he did not even have a degree, has to be withdrawn a little later when he admits that he did complete his PhD before going to Los Alamos.

Kistiakowsky's reminiscences form the most substantial part of the book. He

describes, in non-technical language, the work of his division in designing and making the "implosion" device. He and his collaborators, whose contributions are individually acknowledged, had to fight on two fronts: against professional engineers who insisted on using traditional methods for unprecedented problems, and against physicists who wanted to verify the functioning of the devices by clever, but ambitious and time-consuming, and not always reliable, tests. His lecture ends with a disarmingly frank account of how he had a little too much drink in celebrating VJ Day, and how he fired 21 high-explosive bursts in place of a 21-gun salute, only it came out to be 22.

John Manley and Edwin MacMillan describe the early stages in the creation of the laboratory. Joseph Hirschfelder contributes a brief outline of the work of the laboratory with thumbnail sketches of some of its most famous members. Norris Bradbury, director from 1945 to 1970, deals with the transition to a peacetime institution. There is a historical introduction by Lawrence Badash.

A few questions after each talk give the impression that the audience (or at least its more vocal members) followed the lectures less intelligently than the reader will, but they still elicited interesting answers.

All in all, this mixture of styles and approaches adds up to a lively picture of an unusual, and maybe unique, experience. □

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One man's view of desertification

Peter D. Moore

Land, Man, and Sand. By James Walls. Pp.336. (Macmillan, New York/Collier Macmillan, London: 1980.) \$19.95, £12.95.

"WHAT? Not *another* world conference?" Barbara Ward exclaimed in *The Economist* on hearing of the proposed United Nations Conference on Desertification in 1977. "What? Not *another* book reporting the outcome of another world conference", we may be forgiven for retorting on being faced with *Land, Man, and Sand*, a summary of the Desertification Conference's findings.

It is, however, a slightly different approach to the need for disseminating the results of such conferences in that it is

written by a single author, and he an experienced writer. Instead of being presented with an uncoordinated, turgid set of papers, we have a unified, flowing account which draws liberally upon the original reports and documents. It is an interesting experiment in communication, the main disadvantage of which is that we are inevitably given one man's viewpoint, however fair and objective that man may attempt to be.

If there is one thing more boring than collections of conference reports, it is accounts of how conferences come to be convened. No doubt much thoughtful, bureaucratic effort goes into this area of scientific communication but, generally

speaking, it lacks the glamour or even scientific content which warrant an introductory chapter. The only vital information within it is that the core of the conference was to be comprised of 15 case studies of desert-spread research from widely separated parts of the world. It is these studies which have formed the basis for the remainder of this book.

There are two aspects of the character of James Walls which emerge from these chapters and which demand comment. The first is that he handles language skilfully, though some of his descriptive passages would be more appropriate in a novel than in a supposedly scientific text. The second is that he is eager to comment upon rather than simply to paraphrase the reports which form the basis for the book. His comments are generally critical, incisive, voluble and often completely lacking in any factual basis. He is a master of emotive writing and selective editing.

There is a danger here of falling into the same trap, so I will confine my specific comments to the one case study in which I have had first-hand experience, namely the Turan study of north-east Iran. Walls' report of this study contains a regular smattering of factual errors. He constantly equates the modern town of Shahroud with the Parthian capital Hecatompylos; more important, his reproduced maps confuse areas of moving sands with the saline plains, thus making a nonsense of subsequent discussion of the geography

and sociological effects of sand movements.

It is this kind of betrayal of his lack of knowledge of the places and the people in this study which leads one to take with a pinch of gypsum his hostile comments about the sociological base of the study. For example, the statement is made in the case study that the traditional pastoralist of Turan is concerned more with the condition of his animals than with that of the vegetation. This conclusion is regarded as vapid and patronizing by Walls. How he can level such criticisms without the necessary experience is difficult to discern. The sociologist/anthropologists involved in this study spent many months living with the people of Turan and spoke fluent Farsi. I would prefer to trust their judgement on the issue than the preconceptions voiced by the commentator.

Sadly, the book exhibits many such examples of the author expressing his opinion in areas where it is unlikely that he has experience to equal the originators of the case study reports. These reports incidentally, are available in complete form, free from editorial comment (held by the United Nations Environmental Programme) and I would strongly recommend interested parties to seek these for information rather than this book. □

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Who's playing games?

Lindon J. Eaves

The IQ Game: A Methodological Inquiry into the Heredity-Environment Controversy. By Howard F. Taylor. Pp.276. (Harvester/Rutgers University Press: 1980.) £18.95, \$19.50.

The IQ Game arises from the author's "Sincere concern with the accurate assessment of the causes of variation in IQ". It is a detailed development of three principal criticisms of past research into the genetic basis of intelligence. The author's concern primarily with "heritability" and "IQ" may have limited his opportunity to explore in detail many other relevant aspects of research and theory in the general area of human quantitative genetics.

Dr Taylor's first two arguments have a familiar flavour to those who have followed the discussions of the past 15 years: (i) data are inadequate; (ii) parameters estimated from only one or two kinship correlations may be biased. The third main theme is an assault on the case where it is strongest — in the work of the path analytic and biometrical genetical schools whose long campaign to raise the standard of debate in the area is at last bearing fruit. Even here, where large numbers of kinship correlations are examined simultaneously with explicit models and statistical tests, the author suggests that the possibility of a very substantial environmental component cannot be excluded. In developing this position he relies heavily on a single model for the genetic and social transmission of intelligence suggested early in the 1970s by Morton and his associates, with its peculiarly idiosyncratic assumptions about the mating system and environmental transmission. The book depends also on the cogent criticisms and corrections of Professor Goldberger. It is convenient to have these reviewed in a more accessible form.

Dr Taylor shows that the remarkable consistency of multiple kinship data with a simple model for genetic and social transmission might be improved still further by the inclusion of numerous additional "environmental" components to explain anomalies in the data. Taken to the limit, indeed, it is possible to dispense entirely with a genetic component by recourse to as many *ad hoc* environmental parameters as there are kinship correlations to be explained. Those who play the "IQ game", according to Dr Taylor, make such arbitrary and implausible assumptions as will secure an estimate of heritability. This leads to the claim that a "plausible" relaxation of assumptions would reduce the estimated contribution of genetic factors and justify complete agnosticism about the causes of individual differences. In his analysis, however, relaxation of

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assumptions is not accompanied by any reduction in residual degrees of freedom. He is thus proceeding as if *any* set of environmental correlations is equally appealing.

It is here that the author's basic misconception of the "game" is revealed. However much the assumptions of the Hawaii model might be questioned on biological or psychological grounds, they are nevertheless founded upon a strong yet simple theory of genetic and social transmission. Such a theory comes close to predicting the observed distribution of individual differences in IQ. Science only degenerates into a "game" when additional components are imported *post hoc* with no clear basis in a theory of gene action or social interaction.

The provision of competing environmental theories which could match the classical genetic theory for simplicity and explanatory power is an exciting and challenging prospect. The book devotes three pages to a number of positive suggestions, of which the need to develop

environmental theories is but one. It is suggested that IQ should be treated as an index of an underlying construct rather than the actual phenotype of interest; that models for multiple cognitive measures be developed; that models and designs should be explored to allow separation of the developmental recursion of genetic and environmental factors.

Had these suggestions been published at the start of the past decade they would have been heralded as a positive and imaginative contribution. Now, however, Dr Taylor is not alone in his concern for truth. A substantial body of theoretical and empirical research in the wider area of human and behavioural genetics has been published in journals and conference proceedings. Over the past five years a significant start has been made on the study of precisely these issues and in response to those criticisms that the book develops in such detail. □

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Complexity of melting

G. Gee

Crystal Melting. Macromolecular Physics, Vol.3. By Bernhard Wunderlich. Pp.363. (Academic: 1980.) \$42.50, £23.80.

THIS volume, the third in the author's treatise *Macromolecular Physics*, adds three chapters to the seven already published (in 1973 and 1976). The three volumes deal successively with the description of macromolecular crystals, with their growth and now with their melting. Although the author states that the first part of the project is now complete the next volume is evidently planned in some detail, for there are numerous references forward to it for thermodynamic discussions which are needed in this volume. This spreading of intimately interconnected topics over four volumes and ten years is a necessary consequence of a single author undertaking such a huge task, but it is not very helpful to today's reader to be told (p.104): "the derivation is obvious by comparison with the results listed in 11.2.3" (Vol.4).

In any elementary thermodynamics course melting is presented as a simple example of equilibrium between two bulk phases: a perfect crystal and its melt. Why then a whole volume on melting? In the first place, polymer crystals are characteristically small and imperfect: 100 per cent crystallinity is seldom attainable. Even the "melt" may require further specification, for example molecular weight and perhaps cross-linking or (in copolymers) composition. Finally, equilibrium between the two is in most cases almost impossible to achieve. These problems are recognized in the chapter divisions: equilibrium melting; irreversible melting; copolymer and isomer melting. In each chapter a discussion of general principles is followed by a detailed and critical survey of what is considered to be the most important and reliable experimental work.

This volume, like its predecessors, is addressed primarily to the specialist reader. He can safely be assumed to have at least a general knowledge of the topics discussed in other volumes (including those yet to appear). For him it is a splendid book, a reliable guide to the literature, offering a considered judgement but quick to point out difficulties. It is altogether too detailed for a reader wanting only a general account of this aspect of polymer physics. Between the two extremes will be many, engaged perhaps in the making or application of polymers, who will find it a useful source of reference and comment on the behaviour of a particular material.

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The second edition of Gregory Bateson's *Naven*, the classic study of the culture of the Iatmul people of New Guinea, has been reissued by Wildwood House, price £4.50.

How microorganisms get their nitrogen

Ben Mifflin

Microorganisms and Nitrogen Sources: Transport and Utilization of Amino Acids, Peptides, Proteins, and Related Substrates. Edited by J.W. Payne. Pp.870. (Wiley: 1980.) £45, \$126.60.

THE lengthy sub-title of this book is intended, according to the editor, to provide a more complete indication of the coverage of the volume. However, even this does not really do justice to the number of topics covered. For example, at least four of the chapters deal with some aspect of nitrogen metabolism in higher plants, one of which is entirely devoted to the subject. Further, although the book set out to cover transmembrane metabolism, it also deals in some detail with the utilization of ammonia, amino acids and peptides. In all there are nearly 900 pages, spread over 26 contributions which are divided into four sections: Amino Acids, Peptides, Proteins, Related Systems. These have been written by a variety of international authors, many prominent in their field. Six chapters cover the transport and utilization of amino acids and peptides in bacteria, yeast and fungi. The genetics, regulation and energetics of the uptake of these nitrogen sources are dealt with separately in a further six chapters. Reflecting our greater knowledge of bacterial biochemistry, relative to that of other organisms, there is discussion of the membrane proteins responsible for amino acid transport. The section on proteins, although still dealing with transport, is more heavily biased towards utilization and re-utilization.

The final section of the book is to me what makes it really worthwhile; it is unusual in that the editor has included a number of contributions that should encourage the readers to broaden their horizons. Thus, there is a chapter on the γ -glutamyl cycle in animals and speculation as to how it might apply to microorganisms, and two on the utilization of nitrogenous compounds by green autotrophic plants. The utilization of ammonia and amides is well covered in three contributions and the reader is finally informed of some of the other functions of peptides, notably as antimicrobial agents and ionophores.

The contributions have generally been written with the aim of making them a readable and developmental account of the field rather than a combination of the author's latest results and his reference card library which, alas, all too often occurs in multi-authored volumes. The coverage of the literature is generally broad although not exhaustive (or exhausting) and, considering the size and number of authors, is reasonably up to date.

Whilst it is possible to argue with various points expressed and to pick out certain mistakes, this would be ungenerous to an editor and authors who have obviously striven hard to provide an informative and stimulating book, which I am sure will be a standard work in the field for many years to come. □

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Aspects of the oceanography of shelf seas

Henry Charnock

The North-West European Shelf Seas: The Sea Bed and the Sea in Motion. Vol. II, Physical and Chemical Oceanography, and Physical Resources. Edited by F.T. Banner, M.B. Collins and K.S. Massie. Pp.337. (Elsevier Scientific: 1980.) Dfl.185, \$90.25.

IN THIS addition to the Elsevier Oceanography Series, five specialists deal with the tides, the wave climatology and with the physical oceanography of the Irish Sea, the North Sea, and the Celtic Sea and English Channel. One chapter deals with tidal power and a further seven are devoted to trace metals, to artificial radioisotopes, to sediment transport and to chemical and seabed resources (other than hydrocarbons). Volume I of this two-volume set dealt with the geology and sedimentology of the same area.

The physical oceanography contributions (Irish Sea by Bowden, Celtic Sea and English Channel by Pingree, and North Sea by Lee) are complementary, treating the same types of phenomena from different points of view. Coastal oceanography is clearly more complicated, if not more difficult, than that of the deep ocean: conditions are much less homogeneous so that it is difficult to study idealized processes. But the economic and aesthetic

importance of some of the problems has led to an increase in the effort being put into trying to solve them: the numerical model succinctly described by Pingree should prove an increasingly valuable tool. Similar models are referred to by Huntley in his scholarly chapter on tides.

Of the other chapters, those on wave climatology (Draper), trace metals (Burton, Young) and artificial radioisotopes (Mauchline) are essentially factual. It is especially in these chapters that the delay between the preparation of the book and its publication is to be regretted; new measurements are being made, and old results updated, at a gratifyingly rapid rate.

Contributions on sediment transport and seabed resources (both by Banner) and the extraction of inorganic materials (Massie) illustrate the possibilities for exploitation of the ocean. They should be read in the context of the other chapters and in particular with that on socio-

economic development and the management of coastal zones, by Harding, which gives a good account of how difficult it is to use the ocean wisely, even (especially?) in a confined region bounded by developed nations. The north-west European shelf seas (and perhaps especially the North Sea) represent a microcosm of what is increasingly recognized as a global problem of the future.

Since this region is one of the relatively few with large tidal range, it was perhaps sensible to include a chapter on tidal power (by Wilson). This gives a clear account of the Rance Scheme, of the USSR experience and of the UK prospects, but could be better appreciated if there was a related account of wave energy, and perhaps of wind power.

There is, one supposes, rather little in this book which could not be found elsewhere, but it is convenient to have the material gathered together into one volume. □

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Mycotoxin biosynthesis reviewed

E. Arthur Bell

The Biosynthesis of Mycotoxins: A Study in Secondary Metabolism. Edited by Pieter S. Steyn. Pp.432. (Academic: 1980.) \$44, \$24.80.

WIDESPREAD interest in the mycotoxins dates from the discovery of the aflatoxins in the early 1960s. Since then much has been published on these compounds and, as Sir Derek Barton states in his foreword to this volume, "the time is certainly ripe for an authoritative book". The present "authoritative book" is a collection of chapters written by leading workers in the field.

In the first chapter Bu'Lock discusses mycotoxins as secondary metabolites derived variously from polyketides, peptides, terpenes and other precursors. He emphasizes, moreover, that secondary metabolism, including mycotoxin biosynthesis, can be affected dramatically not only by the genetic modification of a fungus but also by the conditions under which the organism is grown or has been grown in the past. In succeeding contributions specific groups of mycotoxins are dealt with in detail. The biosynthesis of lysergic acid and the ergolines in *Claviceps* species is the subject of a contribution by Floss and Anderson. The formation of tricothecenes from mevalonate via farnesyl pyrophosphate in the Fungi Imperfecti is discussed by Tamm and Breitenstein, while Steyn, Vleggaar and Wessels describe the various techniques which have been used to elucidate the pathways of aflatoxin

synthesis from polyketide precursors.

The ergochromes, also of polyketide origin, are dealt with by Franck, while Yamazaki provides us with information concerning a heterogeneous collection of neurotropic mycotoxins, including aspergillilic acid, which is formed from leucine and isoleucine, and slaphramine, a lysine derivative.

The biosynthesis of patulin and penicillic acid is considered by Zamir and that of the cytochalasans, a group of mixed biosynthetic origin, by Tamm. Kirby and Robins write of gliotoxin and related compounds, Holzapfel of cyclopiasonic acid, and Sankawa of the anthraquinonoid mycotoxins. The final chapter by Vleggaar and Steyn on "miscellaneous mycotoxins" provides even more evidence of the diversity of the biochemical pathways which lead to these fungal products.

It is a pity that the reader is given so little information about the physiological effects produced by these compounds and the nature of the organisms to which they are toxic. Biosynthesis is, however, the proclaimed subject of the book and the editor and authors have provided us with a veritable mine of information on the subject. This book will prove invaluable to all chemists, biochemists and biologists interested either in secondary metabolism or in the toxins of the fungi. □

E. Arthur Bell is Professor of Biology at King's College, University of London.

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